

Science

8 November 2013 | \$10





page 684



page 696

EDITORIAL

- 670 Ensuring Success for J-NIH
Takashi Kadowaki

NEWS OF THE WEEK

- 676 A roundup of the week's top stories

NEWS & ANALYSIS

- 679 Israel's Silent Polio Epidemic Breaks All the Rules
680 California Moves Shake Up Prenatal Gene Testing Market
681 Orbiting MAVEN Mission Set to Trace a Planet's History in Thin Martian Air
682 Soldier-Scientists Join Counterinsurgency in Afghanistan
683 The Ears Have It: First Snakes Were Burrowers, Not Swimmers
683 Has Program to Rotate Scientists at NSF Spun Out of Control?

NEWS FOCUS

- 684 The Forgotten Malaria
Malaria as Lifesaving Therapy
>>> *Science Podcast*
688 In the Hot Seat
LETTERS
691 In Defense of WHO's Blood Donation Policy
N. Dhingra
Response
N. Lacetera et al.
Returning to the Colombian Amazon
E. P. Anderson and J. A. Maldonado-Ocampo

- 693 CORRECTIONS AND CLARIFICATIONS

BOOKS ET AL.

- 694 Play, Playfulness, Creativity and Innovation
P. Bateson and P. Martin, reviewed by G. R. Brown
695 Europa Report
S. Cordero, director;
Gravity
A. Cuarón, director, reviewed by S. Hameed

POLICY FORUM

- 696 Hell and High Water:
Practice-Relevant Adaptation Science
R. H. Moss et al.
>>> *Science Podcast*

PERSPECTIVES

- 699 Fusion for Moving
G. A. Clawson
700 A Stem Cell Perspective on Cellular Engineering
S. Doulatov and G. Q. Daley
702 Quantum Mechanics Tackles Mechanics
K. Hammerer
>>> *Report p. 710*
703 Cold-Atom Thermoelectrics
T. T. Heikkilä
>>> *Report p. 713*
705 Genetics Driving Epigenetics
T. S. Furey and P. Sethupathy
>>> *Reports pp. 744, 747, and 750*
706 Have Your PIC!
S. Malik and R. G. Roeder
>>> *Research Article p. 709*

ON THE WEB THIS WEEK

>> Science Podcast

Listen to stories on characterizing a meteorite, using science to adapt to climate change, the other malaria, and more.

>> Find More Online

Check out Science Express, our podcast, videos, daily news, our research journals, and Science Careers at www.sciencemag.org.



COVER

Raman mapping images of carbon isotope-labeled graphene domains (lateral dimensions: 50 to 120 micrometers) created by exposing a copper surface to normal and isotope-labeled methane at preselected intervals. The data provide information about the effect of growth parameters on the domain shape and size and also contribute to the growth of centimeter-size single crystals of graphene that could enable applications such as nanoelectronics, photonics, and ultrastrong materials. See page 720.

Image: Yufeng Hao

DEPARTMENTS

- 669 This Week in Science
671 Editors' Choice
674 Science Staff
753 New Products
754 Science Careers

REVIEW

- 708** Epithelial Plasticity: A Common Theme in Embryonic and Cancer Cells
M. A. Nieto

Review Summary; for full text:

<http://dx.doi.org/10.1126/science.1234850>

RESEARCH ARTICLE

- 709** Architecture of an RNA Polymerase II Transcription Pre-Initiation Complex
K. Murakami et al.

The yeast transcription pre-initiation complex has a bi-lobed structure that may reflect the assembly pathway of the complex.

Research Article Summary; for full text:

<http://dx.doi.org/10.1126/science.1238724>

>> Perspective p. 706

REPORTS

- 710** Entangling Mechanical Motion with Microwave Fields
T. A. Palomaki et al.

Quantum entanglement is demonstrated between a macroscopic mechanical oscillator and a microwave field.

>> Perspective p. 702

- 713** A Thermoelectric Heat Engine with Ultracold Atoms
J.-P. Brantut et al.

A flow of particles in response to a thermal gradient is observed in a channel connecting two reservoirs of ^6Li atoms.

>> Perspective p. 703

- 716** Visualization and Quantification of Electrochemical and Mechanical Degradation in Li Ion Batteries
M. Ebner et al.

Synchrotron x-ray tomography can be used to study failure modes in an operating battery.

- 720** The Role of Surface Oxygen in the Growth of Large Single-Crystal Graphene on Copper
Y. Hao et al.

Oxygen treatment of a copper surface promoted the faster growth of compact, centimeter-scale graphene domains.

- 724** Asymmetric Distribution of Lunar Impact Basins Caused by Variations in Target Properties
K. Miljković et al.

Numerical simulations imply that lunar impact basins are not representative of the earliest inner solar system impact flux.

- 727** T_H17 Cell Differentiation Is Regulated by the Circadian Clock
X. Yu et al.

Diurnal regulation of an immune cell lineage in the intestine protects against inflammatory disease in mice.

- 731** High-Resolution Mapping of the Spatial Organization of a Bacterial Chromosome
T. B. K. Le et al.

A bacterial chromosome is organized into self-interacting regions delimited by highly expressed genes.

- 734** Mitochondrial Fusion Directs Cardiomyocyte Differentiation via Calcineurin and Notch Signaling
A. Kasahara et al.

Interrupting mitochondrial fusion inhibits cardiac differentiation by dysregulating a specific cell signaling pathway.

- 737** The Hippo Signaling Pathway Interactome
Y. Kwon et al.

A proteomics approach for protein-protein interactions reveals new components of a conserved cell signaling pathway.

- 741** High-Speed Force Spectroscopy Unfolds Titin at the Velocity of Molecular Dynamics Simulations
F. Rico et al.

Experimental time scales previously accessible only to simulations provide insight into forced protein unfolding.

- 744** Coordinated Effects of Sequence Variation on DNA Binding, Chromatin Structure, and Transcription
H. Kilpinen et al.

Human genetic variation results in coordinated allelic variation across molecular phenotypes.

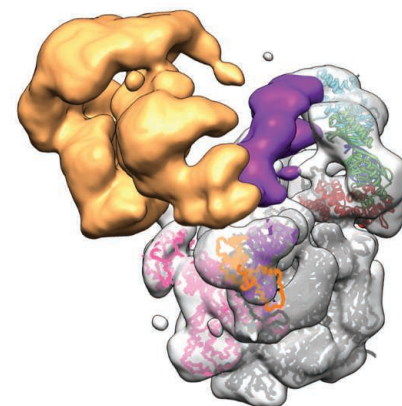
- 747** Identification of Genetic Variants That Affect Histone Modifications in Human Cells
G. McVicker et al.

Human genetic variation affects transcription factor binding, leading to histone modifications.

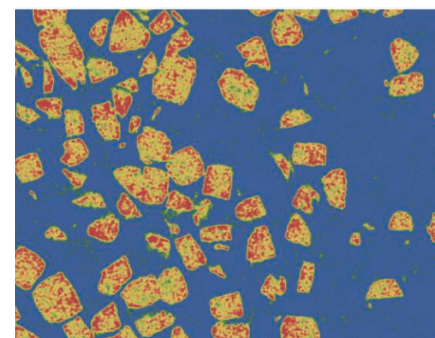
- 750** Extensive Variation in Chromatin States Across Humans
M. Kasowski et al.

Variability among humans with different ancestry affects chromatin states and gene expression.

>> Perspective p. 705



pages 706 & 709



page 716

SCIENCE (ISSN 0036-8075) is published weekly on Friday, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue, NW, Washington, DC 20005. Periodicals Mail postage (publication No. 484460) paid at Washington, DC, and additional mailing offices. Copyright © 2013 by the American Association for the Advancement of Science. The title SCIENCE is a registered trademark of the AAAS. Domestic individual membership and subscription (51 issues): \$149 (\$74 allocated to subscription). Domestic institutional subscription (51 issues): \$990; Foreign postage extra: Mexico, Caribbean (surface mail) \$55; other countries (air assist delivery) \$85. First class, airmail, student, and emeritus rates on request. Canadian rates with GST available upon request, GST #1254 88122. Publications Mail Agreement Number 1069624. Printed in the U.S.A.

Change of address: Allow 4 weeks, giving old and new addresses and 8-digit account number. Postmaster: Send change of address to AAAS, P.O. Box 96178, Washington, DC 20090-6178. Single-copy sales: \$10.00 current issue, \$15.00 back issue prepaid includes surface postage; bulk rates on request. Authorization to photocopy material for internal or personal use under circumstances not falling within the fair use provisions of the Copyright Act is granted by AAAS to libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that \$30.00 per article is paid directly to CCC, 222 Rosewood Drive, Danvers, MA 01923. The identification code for Science is 0036-8075. Science is indexed in the Reader's Guide to Periodical Literature and in several specialized indexes.



<< Dissecting Hippo Interactions

The Hippo signaling pathway plays key roles in many processes, from cell proliferation and cell death to regulation of stem cells and cancer cells. **Kwon *et al.*** (p. 737, published 10 October) attempted to systematically identify all components of the pathway. A protein-protein interaction screen identified more than 200 interactions among approximately 150 proteins. A protein identified in the screen, Leash, restrained the activity of the transcriptional coactivator Yorkie, which regulates gene expression in response to Hippo signaling.

Optomechanically Entangled

Quantum entanglement allows engineered quantum systems to exceed classical information processing bounds. **Palomaki *et al.*** (p. 710, published online 3 October; see the Perspective by **Hammerer**) extend this quantum resource into the domain of micromechanical oscillators by demonstrating entanglement between a microwave field and a mechanical oscillator. The mechanical part of the entangled state could then be transferred to a second microwave field.

Battery Breakdown

Although a range of materials can be used for chemically storing electrical charge, many cannot be made into batteries that retain their capacity over many cycles. Failure may be because of secondary reactions, poisoning through the formation of surface coatings, or volumetric changes leading to fracture. **Ebner *et al.*** (p. 716, published online 17 October) studied this last scenario in an operating battery using synchrotron x-ray tomographic microscopy, tracking both the chemical changes in the battery and the resulting mechanical changes in a tin oxide model system, which is known to undergo large volume changes.

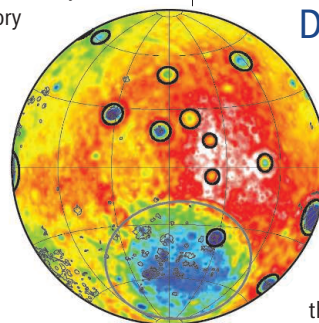
Oxygen Control of Graphene Growth

The growth of graphene on copper surfaces through the decomposition of hydrocarbons such as methane can result in a wide variety of crystal domain sizes and morphologies. **Hao *et al.*** (p. 720, published online 24 October; see

the cover) found that the presence of surface oxygen could limit the number of nucleation sites and allowed centimeter-scale domains to grow through a diffusion-limited mechanism. The electrical conductivity of the graphene was comparable to that of exfoliated graphene.

Which Side of the Moon?

The far- and nearsides of the Moon are geologically different. Using high-precision crustal thickness maps derived from NASA's Gravity Recovery and Interior Laboratory (GRAIL) mission, **Miljković *et al.*** (p. 724) show that the distribution of lunar impact basins is also highly asymmetrical. Numerical simulations of impact basin formation coupled with three-dimensional simulations of the Moon's asymmetric thermal evolution suggest that lateral variations in temperature within the Moon's crust have a large effect on the final size of an impact basin.



Lighting Up Immunity

T_H17 cells are $CD4^+$ T helper cells that produce the proinflammatory cytokine interleukin-17. In the intestines, T_H17 cells protect the host from fungal and bacterial infections, and their proinflammatory function is linked with autoimmune diseases including inflammatory bowel disease. **Yu *et al.*** (p. 727) show that the molecular circadian clock directly regulates the differentiation of T_H17 cells in the intestine, which suggest that both nutrition and light are important environmental factors that directly regulate the immune response.

Mitochondrial Fusion and Heart Development

The role of mitochondria in fueling homeostatic cell processes is well appreciated, but whether and how they influence cell differentiation is much less clear. Using *in vivo* embryonic mouse models and mouse embryonic stem cell cultures, **Kasahara *et al.*** (p. 734, published online 3 October) found that an intact mitochondrial fusion pathway was essential for the cellular signaling that drives mesodermal cell differentiation into cardiomyocytes.

Bridging the Titin Gap

The muscle protein titin is a molecular spring that has been extensively studied by single-molecule unfolding experiments and by molecular simulation. However, experimental and simulated unfolding could not be compared directly because they differ by orders of magnitude in pulling velocity. **Rico *et al.*** (p. 741) developed high-speed force spectroscopy to pull titin molecules at speeds that reach the lower limits of molecular dynamics simulations. Bridging the gap between simulation and experiment clarified the mechanism of conformational changes in titin.

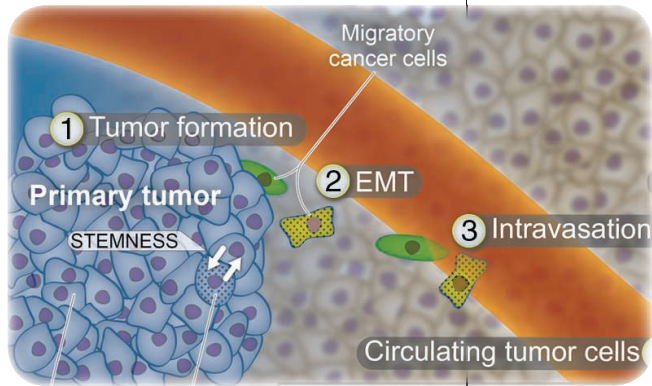
DNA Differences

The extent to which genetic variation affects an individual's phenotype has been difficult to predict because the majority of variation lies outside the coding regions of genes. Now, three studies examine the extent to which genetic variation affects the chromatin of individuals with diverse ancestry and genetic variation (see the Perspective by **Furey and Sethupathy**). **Kasowski *et al.*** (p. 750, published online 17 October) examined how genetic variation affects differences in chromatin states and their correlation to histone modifications, as well as more general DNA binding factors. **Kilpinen *et al.*** (p. 744, published online 17 October) document how genetic variation is linked to allelic specificity in transcription factor binding, histone modifications, and transcription. **McVicker *et al.*** (p. 747, published online 17 October) identified how quantitative trait loci affect histone modifications in Yoruban individuals and established which specific transcription factors affect such modifications.

Additional summaries

From Here to There

To form different tissues and organs, embryonic cells must migrate to new locations. Specific transcription factors, epigenetic and splicing programs, and microRNA regulatory networks regulate this process, which is known as the epithelial-to-mesenchymal transition (EMT). During EMT, considerable cellular plasticity is



observed, and once activated at their new location, cells must again change into their new differentiated form. This “reverse” event is called the mesenchymal-to-epithelial transition (MET). **Nieto** (p. 708) reviews EMT and MET as observed during normal development and in the generation of cancer when cells leave the primary tumor and travel to other parts of the body forming metastases and secondary tumors.

Pre-Initiation Complex in 3D

The regulation of gene expression is critical for almost every aspect of biology. Transcription—generating an RNA copy of a gene—requires the assembly of a large pre-initiation complex (PIC)

at every RNA polymerase II (pol II) promoter. Roughly 32 proteins—the subunits of pol II and the general transcription factors—form a PIC that can recognize a minimal TATA-box promoter, select a transcription start site, and synthesize a nascent transcript. **Murakami et al.** (p. 709, published online 26 September; see the Perspective by **Malik and Roeder**) determined

the three-dimensional map of the *Saccharomyces cerevisiae* 30-subunit PIC using cryo-electron microscopy. The saddle-shaped TATA binding protein, the boot-shaped transcription factor IIA (TFIIA), and promoter DNA ~27 bp downstream of the TATA-box could all be seen. Cross-linking and mass spectrometry was used to determine the spatial proximity of the 30 subunits, revealing that the PIC forms two lobes with TFIIF forming a bridge between them.

Cold Thermoelectrics

Thermoelectric effects—such as the creation of a voltage drop in response to a thermal gradient (known as the Seebeck effect)—can be used for a number of applications, including converting wasted heat into power. However, especially in solids that exhibit electronic interactions, this type of behavior is not well understood. **Brantut et al.** (p. 713, published online 24 October; see the Perspective by **Heikkilä**) studied the Seebeck effect in the very controllable setting of cold atomic gases. Two initially identical reservoirs of ^6Li atoms were connected using a quasi-two-dimensional channel, and the particle

current after heating one of the reservoirs was measured. The atoms moved from the warmer to the cooler reservoir, the extent of which fit with theoretical predictions as the disorder in the channel and its geometry were varied.

Caulobacter Chromosome

Chromosomal DNA must be highly compacted to fit within the tiny volume of the cell, while at the same time it must maintain a conformation that allows critical cellular processes access to the genome. **Le et al.** (p. 731, published online 24 October) analyzed the structure of the circular chromosome in the prokaryote *Caulobacter crescentus* by using chromosome conformation capture and deep-sequencing. Highly self-interacting regions (chromosomal interaction domains, or CIDs) were observed—similar to the topologically associated domains previously seen in eukaryotes. Supercoiling helped to establish CIDs, and CID boundaries were defined by highly expressed genes. CIDs appeared to be established during or shortly after DNA replication, and could potentially facilitate chromosomal segregation by preventing newly replicated chromosomes from becoming entangled.

Ensuring Success for J-NIH

ON 30 AUGUST 2013, THE JAPANESE GOVERNMENT DECIDED TO RESERVE PART OF ITS 2014 BUDGET for the launch of a control tower for medical research and development (MedR&D), which, modeled after the National Institutes of Health (NIH) in the United States, has come to be known as the Japanese version of NIH (J-NIH). By integrating all MedR&D projects currently run by the government's compartmentalized ministries, the new institution is expected to ensure strategic budget allocations and facilitate a seamless flow from basic biomedical research to clinical applications.

J-NIH is the main feature of the health and medical strategy developed by Prime Minister Abe and his administration, which swept into power in the general election last year. Although Japan has long presented itself as a scientific and technological powerhouse, its basic life science research has often failed to lead to the development of pharmaceuticals or medical devices because of a weak translational process. The government regards a health and medical strategy aimed at promoting longevity and industry competitiveness as an important part of an overall plan for economic growth. But this objective should not come at the expense of basic life science research.

J-NIH will consist of a Health and Medical Strategy Headquarters, headed by Prime Minister Abe, which will develop its overall policies. J-NIH will also include an incorporated administrative agency (IAA) to allocate funds to universities and research institutions. Next year, IAA is expected to control a budget of 138.2 billion yen, an increased investment in MedR&D of 37% from 2013. J-NIH's main projects currently address major diseases facing Japan, such as cancer and dementia; bring the world's cutting-edge medicine, such as induced pluripotent stem (iPS) cell-based regenerative medicine and genomic medicine, to the clinical arena; and reinforce the infrastructure needed to produce innovative pharmaceuticals and medical devices.

The J-NIH concept may be considered a welcome break from ministries long regarded as detrimental to appropriate budget allocation. However, the budget for basic life science R&D remains sub-optimal at less than one-tenth that in the United States. Indeed, the J-NIH anticipated total budget of about 226 billion yen (\$2.3 billion U.S.) does not compare with the NIH 2013 budget of approximately \$30 billion. It is particularly important to note that the U.S. NIH designates more than 80% of its budget to competitive research projects proposed by universities, medical schools, and research institutions,* and approximately half of the U.S. NIH budget is devoted to basic research.† As the history of science shows, innovation thrives on broad-based research that sprouts from a “freewheeling” culture of creative thinking in academia; the iPS cell research of Nobel laureate Shinya Yamanaka and his colleagues is a case in point.

With the overall budget for science research remaining the same, it is a matter of great concern among scientists at universities and research institutions in Japan that J-NIH may be associated with budget cuts for broad basic life science research. This must not happen. Japan has lagged behind Western and Asian countries in relative citations in both clinical and basic research; this has been the result, in part, of dwindling funds for basic life science research.

I strongly urge that both basic life science and translational research be strengthened at the same time in the 2014 budget in conjunction with plans to launch J-NIH. Importantly, as per the RU11 (a consortium of 11 research-intensive universities in Japan) recommendations of 2013, opinion on Japan's investment in science and innovation should continue to be widely sought in the scientific community, reaching out beyond the industrial community and the governmental Council for Science and Technology Policy. My sincere hope is that open discussions on a future J-NIH will touch off a reform of the current compartmentalized institutions that will increase support for both medical and basic life science research at large and allow genuine strategic innovation in translational research.

— Takashi Kadowaki

10.1126/science.342.6159.670

*www.nih.gov/about/budget.htm#note. †www.hhs.gov/budget/budget-brief-fy2013.pdf.



Takashi Kadowaki is director of the Translational Research Initiative at the University of Tokyo and director of the University of Tokyo Hospital. E-mail: kadowaki-3im@h.u-tokyo.ac.jp; kadowaki-dm@umin.net.



ASTRONOMY

Enriched Old Stars

Globular clusters, the oldest surviving stellar systems in galaxies, were for a long time thought to be composed of stars that formed simultaneously with the same initial chemical composition. However, multiple stellar populations with a range of chemical compositions have been observed over the last few years, hinting at a complex and prolonged history of star formation and chemical enrichment. To put constraints on models of globular cluster formation, Schiavon *et al.* studied the correlations between cluster mass and the mean stellar abundances of Fe, Mg, C, N, and Ca for 72 old globular clusters in the Andromeda galaxy—the nearest spiral galaxy to the Milky Way. They found a correlation between N abundance and cluster mass, implying that the chemical enrichment undergone by globular clusters is proportional to their masses. The absence of correlations between mass and Mg and Ca abundances rules out enrichment through explosive nucleosynthesis. — MJC

Astrophys. J. **776**, L7 (2103).

ing. Damage occurs when metastatic tumor cells recruit pre-osteoclast cells to the bone and then induce their differentiation into mature bone-degrading cells, which results in the release of proteins from the bone matrix that promote tumor cell growth. In a study exploring the molecules controlling osteoclast differentiation in the cancer setting, Ell *et al.* have identified miRNAs (microRNAs are small noncoding RNAs that regulate gene expression) whose levels were consistently down- or up-regulated in differentiating osteoclasts. Treatment in a mouse model of metastatic breast cancer with two of the down-regulated miRNAs (miR-141 and miR-219) suppressed bone metastases, suggesting that these miRNAs may have therapeutic utility. The up-regulated miRNAs proved interesting for a different reason: Two of them (miR-16 and miR-378) were detected in the blood of tumor-bearing mice and correlated with metastatic burden, suggesting that they may be useful biomarkers of bone metastases. A preliminary assessment of blood samples from breast cancer patients supported this idea. — PAK

Cancer Cell **24**, 542 (2013).

MOLECULAR BIOLOGY

Polycomb Promiscuity

Turning genes off when they are not needed is as important as turning them on when they are. The polycomb repressive complex 2 (PRC2) shuts down gene expression during development in multicellular eukaryotes, and aberrant regulation of it can contribute to cancer. PRC2 is recruited to repress specific genes via its interaction with long noncoding RNAs (lncRNAs) such as HOTAIR and RepA. Other evidence suggests that PRC2 can interact with very many RNAs, and Davidovich *et al.* have extended these latter observations to show that, in vitro, PRC2 can bind RNA promiscuously and with affinities similar to those of its specific interactions with HOTAIR and RepA. In an analysis of previously published data sets, they demonstrate that PRC2 can interact with both specific lncRNAs and with RNA transcripts from many highly expressed genes in vivo. Although many of the genes that PRC2 interacts with include repressive chromatin marks, as expected given its silencing activity, up to one-third contain only activating marks. The capacity to bind a variety of RNAs enables PRC2 to scan the chromatin of active genes for repressive marks, which would indicate that the genes have somehow escaped silencing, and to shut them down again. — GR

Nat. Struct. Mol. Biol. **20**, 10.1038/nsmb.2679 (2013).

EDUCATION

When Neuroscience Guides Education

How do findings in neuroscience guide educational research and practice? In particular, does providing information about the neurobiology of learning to in-service teachers improve their pedagogy? Dubinsky *et al.* addressed these questions by organizing three sessions of BrainU, a summer professional development workshop designed by the Society for Neuroscience, where plasticity was the key neuroscience concept taught. It was expected that teachers would see themselves as capable of changing students' neural circuits and feel empowered by being able to explain why practice and application were needed to consolidate learning. BrainU provided inquiry-based activities that teachers could subsequently use with their students. A multiple-choice test given before and after the workshop showed that teachers' basic neuroscience knowledge improved. Two pedagogical quality tests given to teachers once they returned to their classrooms indicated that cognitive engagement among students and teachers improved. Thus, BrainU succeeded in motivating teachers to implement student-centered lessons, based

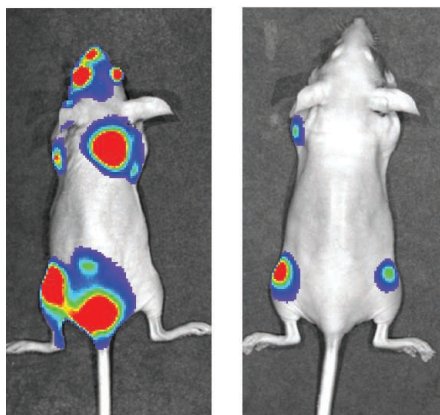
on the concept of plasticity, and in modeling best practice in inquiry pedagogy. This research raises the question of whether teaching the neurobiology of learning to preservice teachers is also efficient. — FB

Edu. Researcher 10.3102/0013189X13499403 (2013).

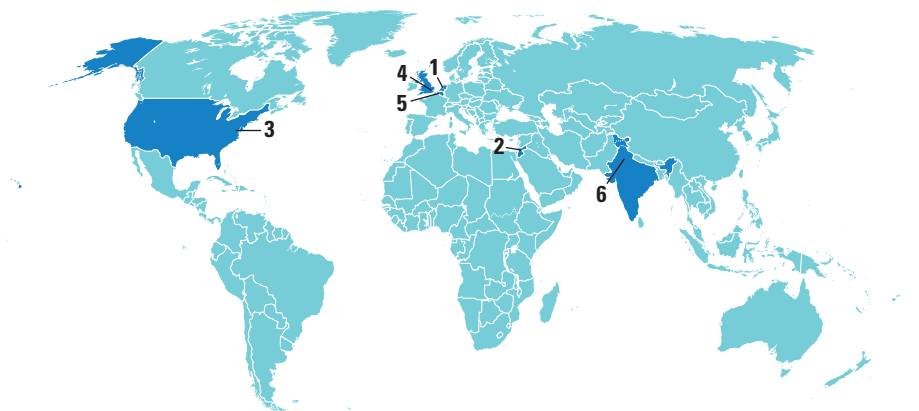
CANCER

Preserving Bones

The most common site of metastatic disease in women with advanced breast cancer is bone. These lesions are painful and can be debilitating.



AROUND THE WORLD



Rotterdam, the Netherlands 1

Virologist Appeals Decision On H5N1 Export Rules

The legal battle continues between virologist Ron Fouchier and the Dutch government over the publication of research on the H5N1 avian influenza virus. On 1 November, Fouchier's employer, Erasmus MC in Rotterdam, formally appealed a ruling by a Dutch court that the government could limit Fouchier's freedom to publish his work with the virus. The issue will now come before Amsterdam's Court of Appeal.

Erasmus MC had sued over the government's demand that Fouchier apply for an export license before submitting a paper to *Science*, published in 2012, that described how a few mutations can make H5N1 transmissible between ferrets. On 20 September, the district court in Haarlem ruled that the government had correctly interpreted an E.U. regulation seeking to slow the proliferation of weapons of mass destruction.

The European Society for Virology (ESV) has sided with Fouchier. In a letter to European Commission President José Manuel Barroso, ESV President Giorgio Palù warns that "this Dutch ruling may have far-stretching implications for research and public and animal health within all EU Member States" and urges a debate on the issue at the European level. <http://scim.ag/Fouchfight>

Amman 2

Banana Disease Reaches Jordan

One of the most damaging diseases of banana plants has spread beyond Southeast Asia. Fusarium wilt, or "Panama disease," is caused by a soil fungus that wiped out banana plantations by the mid-20th century,

until they were replanted with a resistant crop. But 20 years ago, a devastating new variant of the fungus, tropical race 4 (TR4), emerged in Taiwan. It has spread to several countries including China, the Philippines, and Australia.

Now, scientists have confirmed that TR4 is in Jordan. Although only 1500 hectares there are planted with bananas, 80% of the area is infected, underscoring concerns



Lost. Fungus wipes out bananas.

about how easily the pathogen can spread. Researchers from Wageningen University in the Netherlands and other institutions analyzed DNA from fungus samples collected by the Jordanian Ministry of Agriculture and confirmed that they were TR4, the scientists reported online in *Plant Disease*. The researchers called the jump from Asia a "dangerous expansion" of the range and fear that the disease will reach Africa and Latin America, which not only export many bananas but also rely on them for food security.



Understudied. Young athletes' concussions need more research.

Washington, D.C. 3

Report Finds Research Gaps On Youth Concussion in Sports

A report released last week by the Institute of Medicine highlights large gaps in knowledge about sports-related concussions in youth. Most published research on these concussions has been conducted in adults, but it's dangerous to assume those findings can be mapped onto children, because of changes that occur during brain development.

There's not yet enough scientific data to guide safety programs such as the Hit Count Initiative launched last year by the Sports Legacy Institute, which aims to set a threshold for a "safe" number of hits to the head, the report finds. Also unclear is the benefit of preventative measures such as wearing helmets—although they can prevent skull fractures, no available evidence suggests that they prevent concussion. Large, controlled, randomized studies will be needed to answer questions such as whether concussions sustained in childhood can result in long-term problems.

The panel recommends that the Centers for Disease Control and Prevention set up a national surveillance system to monitor the incidence of sports-related concussions, and it recommends that the National Institutes of Health, the Department of Defense, and organizations such as the National Collegiate Athletic Association support research to develop better age-specific recommendations and rules. <http://scim.ag/youthconcussion>

London 4

Personal Genome Projects Spread to Europe

The move to make individual genome sequences—and accompanying personal health information—freely accessible online just got a boost in the United Kingdom. This week, Stephan Beck from University College London and his colleagues announced the establishment of a British Personal Genome Project (PGP), which will recruit

volunteers to provide DNA and health data with no restrictions on their use. This PGP is an outgrowth of a project begun in 2005 by Harvard University's George Church. Often, genome data are freely available, but health information is not because of privacy concerns, making it difficult for researchers to link gene variants to specific conditions. As the PGP coffers grow, researchers will be able to use these data however they need to to inform their disease-gene links studies, Church says. PGP-UK (personalgenomes.org.uk) has funding through the first year to sequence 50 British residents, age 18 or older, and ultimately hopes to enroll 100,000. Already, about 450 have expressed interest, Beck says. All participants have to read and pass a test about a 24-page consent form detailing risks. <http://scim.ag/persgens>

Brussels 5

E.U. Parliament Approves Some Deep-Sea Fisheries Restrictions

After months of delays, the European Parliament's Fisheries Committee approved a report on 4 November to restrict fishing practices deemed destructive for deep-sea ecosystems. But the committee did not back the European Commission's proposal to ban bottom trawling and gillnetting altogether.

Deep-sea trawling fisheries catch 20% to 40% of unwanted fish. About 300 marine scientists signed a petition in favor of the ban, released in June by a French marine conservation nongovernmental organization.

The Fisheries Committee agreed to close off areas harboring vulnerable marine ecosystems to bottom trawling. It also left the door ajar for a general phaseout based on an assessment of the impact of deep-sea fishing gear after 4 years.

The draft legislation will need to be approved by the whole Parliament plenary, possibly in December or January, before negotiations with member states begin. Fisheries ministers will also need to sign off on the plan. <http://scim.ag/deepseatrawl>



Limited. E.U. deep-sea trawling wasn't banned.



Elephants Haunted by Mass Killings

African elephants that have lived through a selective killing, or cull, remain psychologically damaged by the experience for decades, finds a study in the current issue of *Frontiers in Zoology*. Wildlife officials in South Africa culled elephant populations in the 1960s to the 1990s out of fear that they would otherwise destroy the habitat. The officials would save the young elephants 4 to 10 years old from a family and ship them to other parks.

In the new study, researchers compared the reactions of 14 elephant families in Pilanesberg National Park in South Africa (a relocation site) and 39 elephant families in Kenya's Amboseli National Park (a relatively undisturbed population) to social threats. The team would broadcast a call from an unfamiliar older female elephant (possibly another family's matriarch). The Amboseli elephants responded appropriately, with coordinated defensive behaviors, but the Pilanesberg elephants were at a loss, the researchers said—because they never learned the proper behaviors from their own matriarchs. That poor decision-making could be passed down to future generations, affecting reproductive success. <http://scim.ag/elephanthaunt>

New Delhi 6

India Launches Mars Orbiter

India this week sent an orbiter on a journey to Mars. The successful launch of Mangalyaan—meaning “Mars craft” in Hindi—is the culmination of a \$72 million effort by the Indian Space Research Organisation (ISRO). “Capturing and igniting the young minds of India and across the globe will be the major return from this mission,” mission director P. Kunhikrishnan told reporters on 5 November.

Although aimed primarily at showcasing the country's technological prowess, the mission is also supposed to deliver science. The orbiter has six instruments on board that will image the martian surface, sniff the upper atmosphere for traces of methane, and measure the ratio of atmospheric deuterium to hydrogen to understand how the martian atmosphere thinned out over time.

But first the spacecraft must travel more than half a million kilometers over 300 days to get into martian orbit. “The biggest challenge will be precisely navigating the spacecraft to Mars,” K. Radhakrishnan, ISRO's chairman, told reporters. “We will know if we pass our examination on 24 September 2014.”

NEWSMAKERS

Three Q's



Kolodner

Last week, Texas Governor Rick Perry lifted a 10-month moratorium on new grants from the \$3 billion Cancer Prevention and Research Institute of Texas (CPRIT). The freeze followed months

of controversy over improperly reviewed grants that led the agency's chief scientific officer, Nobelist Alfred Gilman, to resign. Heading the overhauled CPRIT's new eight-member Scientific Review Council is Ludwig Institute, San Diego, California, branch cancer geneticist **Richard Kolodner**, the only council member who did not step down last year.

Q: Why did you agree to stay on with CPRIT?

R.K.: Because in this day of declining federal funding for cancer and biological sciences research coming on top of many years of slow degradation of individual NIH [National Institutes of Health] grant budgets, programs that provide significant funding are very important and need to be nurtured. >>

>>NEWSMAKERS

Q: Are you confident that last year's problems won't happen again?

R.K.: I think CPRIT has ... reassessed its leadership and procedures, and is ready to move forward with its mission. ... I have great confidence that [Chief Scientific Officer] Margaret Kripke will build on the tradition of excellence that Al Gilman and [former Scientific Review Council Chair] Phil Sharp established and help CPRIT succeed and continue to fund excellent cancer research.

Q: Will you be able to attract the same caliber of reviewers?

R.K.: Virtually all of the previous reviewers I have contacted have expressed a strong interest in participating as reviewers again.

Frankly I think it is attractive to review grants under circumstances where you know that most if not all of the worthy grants are likely to get funded.

FINDINGS

More Incoming!

February's window-shattering explosion of a meteor over Chelyabinsk, Russia, suggests such encounters are far more frequent than astronomers expected, scientists report this week in *Nature*.

Planetary scientist Peter Brown of the University of Western Ontario in London, Canada, and his colleagues used the Chelyabinsk airburst—observed by video cameras, seismographs, satellite sensors,



Fireball. A witness's photo of the February 2013 Chelyabinsk meteor.

and infrasound detectors—to calibrate the 20-year-long record of airbursts from incoming asteroids. The team found that Earth-impacting asteroids 10 to 50 meters in size have arrived about 10 times the rate estimated from telescopic searches alone.

Planetary astronomer Alan Harris, a NASA consultant in California who came up with that low, search-based estimate, acknowledges that the rate could be several times higher. But 10 times higher? Too few large airbursts like Chelyabinsk, he says, have been detected to persuade him that the threat is *that* great. <http://scim.ag/Chelbursts>

Random Sample

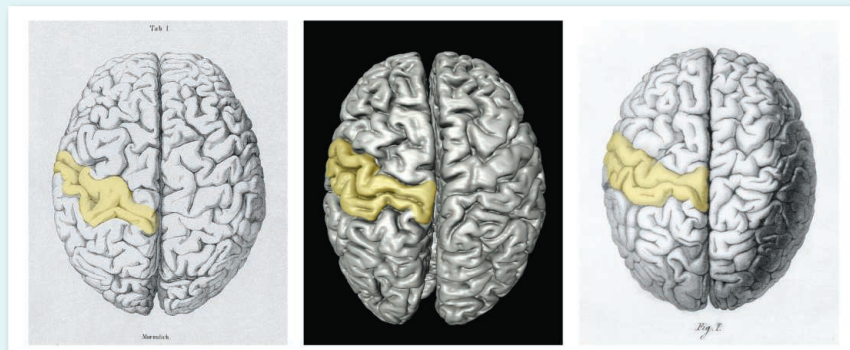
Whose Brain Is It Anyway?

Many scientists have tried to find out where, in the winding topography of the human brain, genius resides. For instance, in the middle of the 19th century, German anatomist Rudolf Wagner collected the brains of numerous professors to study them in detail. The star of the cerebral collection, examined many times in the past 150 years: the brain of German genius and "Prince of Mathematics" Carl Friedrich Gauss.

But when Renate Schweizer of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, recently examined the brain in the University of Göttingen's collection labeled "C. F. Gauss," she noticed a rare anatomical feature in the left hemisphere: a bit of brain tissue bridging the central fissure between the frontal and parietal lobe.

"My first thought was, 'Wow, Gauss had this rare feature,'" Schweizer says. Her second thought was: Wait, I've seen this exact central fissure before.

Her conclusion, published last month in *Brain*: The brain labeled as Gauss's was that of a doctor, Conrad Heinrich Fuchs, whose brain Wagner had also examined. In fact, Wagner had used it to describe for the first time the very anatomical feature Schweizer had noticed. Exactly how and when the brains were mixed up is still unclear, but Schweizer says the culprit was probably Wagner's son Thomas, who compared the brains for his 1864 doctoral thesis—meaning that generations of scientists have examined Fuchs's brain instead of Gauss's. No textbooks will have to be rewritten, however. Anatomically, Gauss's brain is even less exceptional than its long-term substitute, Schweizer says.



Switched. The brains of Conrad Heinrich Fuchs (left and center; fissure in yellow) and Carl Friedrich Gauss (right).

Early Music Lessons Reduce Hearing Loss

It's a well-worn tale of woe: After spending thousands of dollars on music lessons and instruments, parents often watch in dismay as once-coveted flutes, clarinets, and violins are unceremoniously abandoned. Such investments in early musical education aren't wasted, however, a new study in this week's issue of *The Journal of Neuroscience* suggests. Even after going decades without practicing their instruments, adults aged 55 to 76 who studied music for 4 to 14 years when they were young have better "neural timing" than people who never played an instrument, researchers at Northwestern University report.

Neural timing is a type of auditory processing that is key to the ability to interpret speech, and it often declines with age. The more years that participants in the study had spent playing an instrument, the crisper their hearing was even 40 years later, says neuroscientist Nina Kraus, who led the study. Although previous studies have shown that playing music can improve hearing skills, this is the first to show such long-term benefits, she says.

Science LIVE

Join us on Thursday, 14 November, at 3 p.m. EST for a live chat on **robotics and neuro-prosthetics**. <http://scim.ag/science-live>



INFECTIOUS DISEASE

Israel's Silent Polio Epidemic Breaks All the Rules

The first positive sample was detected on 29 May, isolated from sewage in Rahat, a Bedouin community in the south of Israel. Next came a sample from Beer Sheva, a larger southern city that also has a sizable Bedouin population. By July, 30 environmental samples had tested positive, and it was clear that 25 years after it had been dispatched, wild poliovirus was back in Israel and spreading fast.

As of late October, more than 140 specimens from 25 sites had tested positive for poliovirus, mostly in the south but also in central and northern Israel, including Jerusalem and Haifa. Retrospective analysis of sewage samples has shown that the virus has been circulating since February but, surprisingly, has caused no cases of paralysis. Polio outbreaks are raging nearby in Pakistan and war-torn Syria. But in developed, prosperous Israel, no one saw this coming. The country has an impressively high immunization rate of at least 95%, and it uses the inactivated polio vaccine (IPV), the vaccine of choice in high-income countries, which unlike the oral live-virus vaccine used in poorer countries poses no threat of reverting to infectious virus.

Finding circulating wild virus was “totally unexpected,” says Itamar Grotto, director of public health services at the Israeli Ministry

of Health. “[A] shock,” wrote the Independent Monitoring Board of the Global Polio Eradication Initiative (GPEI) in its October report. Not only could the circulating virus spread from Israel to countries now considered polio-free, but it could easily cause cases of paralysis if it infects unvaccinated people in Israel.

The country is now scrambling to quash the silent outbreak before it spreads further. It has also launched a massive scientific investigation to figure out how the virus could be so widespread yet avoid detection. And other wealthy countries, including the United States and the United Kingdom, are watching closely, hoping to draw lessons for their own vaccination and monitoring strategies.

Paradoxically, Grotto says, Israel's high coverage with IPV is what has allowed the virus to circulate silently for so long. In many countries, IPV is the successor to oral polio vaccine (OPV), which has long been the mainstay of the global eradication effort because it is

Outbreak control. As part of the emergency plan, Israeli children are now receiving oral polio vaccine.

cheap and easy to administer in drops. OPV not only protects the individual from paralysis but also induces strong gut immunity that blocks transmission of the virus, which is shed in the stool and spread largely through fecal-oral contamination.

But OPV has a downside. The weakened live virus used in the vaccine can in rare instances regain its neurovirulence and cause paralysis. To avoid that risk, countries that have gotten rid of polio have often switched to the more expensive IPV or a combination of OPV and IPV. Israel adopted an IPV-only schedule in 2005. IPV provides excellent protection against disease, but because it elicits only weak mucosal immunity, it is much less effective than OPV at blocking transmission.

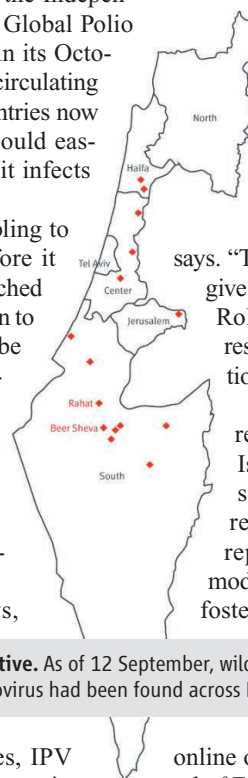
In the face of the virus's reappearance, however, Israel has resurrected the earlier vaccine. After an initial response with IPV, in August Israel began vaccinating all children under age 10 with OPV. In October, the government decided to reintroduce OPV along with IPV into the routine immunization schedule. “Israel has made clear that switching to IPV-only at a time when wild virus is still circulating is a risky strategy,”

says Nicholas Grassly of Imperial College London. It also dispels the notion that IPV by itself might be sufficient to stop an outbreak posteradication. “After Israel, we need to be clear that OPV is the one to use [in outbreaks] and the role for IPV may be limited,” Grassly says. “This episode shows us we should not give up the benefits of OPV lightly,” adds Roland Sutter, who coordinates polio research at the World Health Organization (WHO) in Geneva, Switzerland.

Not all IPV-only countries need to rethink their vaccination strategies. Israel may be a special case, Grotto says—a possibility that Grassly's research suggests. Well before the reports from Israel, he designed a model to see whether IPV could actually foster the spread of the virus in a vaccinated population by masking

an outbreak until it was large and hard to stamp out. In a paper published

online on 7 October in the *American Journal of Epidemiology*, Grassly and colleagues concluded that it could, but only in a “very, very rare” circumstance, Grassly says—when



Positive. As of 12 September, wild poliovirus had been found across Israel.

immunization rates are very high and some portion of the population lives in conditions where the virus transmits very efficiently. “It turns out Israel is one,” says Bruce Aylward, WHO’s assistant director-general who leads GPEI and is a co-author on the paper.

As soon as the virus was detected, Israel expanded its environmental surveillance and began analyzing stool samples to see who was carrying it. They’ve found it predominately in the poor Bedouin population, who mostly live in squalid conditions in the southern part of the country. The children found to be carrying the virus so far have all been fully vaccinated, which is why there have been no cases, but poor sanitation, overcrowding, and migration have enabled the virus to spread.

“It’s a first-world country with a third-

world population,” Sutter says.

Israel’s experience does make clear that every country should have ready access to a supply of OPV to respond to emergencies like this and should use it fast, says Hamid Jafari, director of GPEI at WHO. And countries at risk that don’t monitor the environment for circulating poliovirus—the only way this outbreak was detected—should consider starting, he adds.

Meanwhile, Israeli researchers and their collaborators at WHO and the U.S. Centers for Disease Control and Prevention are trying to figure out where the virus came from. Early phylogenetic analysis linked it to a strain that circulated in Pakistan in 2012, and investigators initially thought it had jumped from Pakistan to Israel via Egypt, where

it was briefly detected in sewage samples late last year. Further sequencing by Lester Shulman of the Ministry of Health’s Central Virology Laboratory in Tel Hashomer suggests a single introduction from Pakistan into the broader region sometime in 2012, after which the virus diverged, with some branches going to Egypt, Israel, and now, presumably, Syria, where an outbreak began in October.

Despite the vaccination campaigns, the virus is still circulating in Israel. The most pressing question now is how much OPV is needed to stop transmission—Israel is gearing up for another national vaccination round with OPV—and how long it will take. “We are inventing the wheel,” Grotto says. “There is no experience to learn from.”

—LESLIE ROBERTS

INTELLECTUAL PROPERTY

California Moves Shake Up Prenatal Gene Testing Market

Two decisions in California last week promise to give a boost to a new technique for prenatal genetic testing, opening the field to increased competition and expanding the market for the tests.

On 30 October, a federal district court judge in San Francisco invalidated a patent owned by Sequenom of San Diego on a novel test for Down syndrome that sieves fetal DNA from a mother’s blood and checks it for risky abnormalities. Three other companies planning to offer similar blood tests, which obviate the need

for taking samples from the womb, now won’t worry as much about being sued over patents. In a separate windfall, California agreed on 1 November to subsidize these fetal DNA tests—known as noninvasive prenatal testing—through the state’s genetic diseases program, which screens about 400,000 women a year.

The decision to nullify Sequenom’s U.S. patent (number 6,258,540) seems to have rattled investors, with the company’s stock price dropping 23% on 31 October. Company executives issued statements saying that they “vigorously” disagree with the ruling, which “misapplies or ignores the controlling law,” and will appeal.

In her opinion, Judge Susan Illston of the federal court for northern California wrote that, in light of recent U.S. Supreme Court decisions, Sequenom should never have

received a patent in 2001 on its prenatal testing methods. They are based on work done in the late 1990s by Yuk-Ming Dennis Lo of the Chinese University of Hong Kong and James Stephen Wainscoat, then of the University of Oxford in the United Kingdom, who developed a way to use paternal DNA to isolate cell-free fetal DNA circulating in a pregnant woman’s bloodstream, making invasive procedures such as amniocentesis unnecessary. The DNA can then be checked for chromosomal abnormalities such as trisomy 21—the cause of Down syndrome.

But Illston wrote that Sequenom’s patent was built on discoveries that for the most part were not very inventive, citing the Supreme Court’s 2009 rejection of a blood test patent held by Prometheus Laboratories of San Diego. Sequenom was trying to claim ownership of lab processes such as amplifying DNA fragments that were already “well-understood” and “routine” when the patent was issued, she wrote. The exception was Sequenom’s novel use of paternal DNA as a screening tool. But DNA is “a natural phenomenon,” she decided, and therefore not patentable. In June, the Supreme Court had denied claims on the *BRCA* breast cancer genes held by Myriad Genetics of Salt Lake City for the same reason, and Illston cited the Myriad decision to buttress her conclusion.

“This will help keep the prices down and provide a driver for general competition.”

—HENRY GREELY,
STANFORD UNIVERSITY

“It’s a huge victory for us and for the entire field,” says Ken Song, CEO of Ariosa Diagnostics of San Jose, California, the firm that started this legal brawl. Ariosa, Sequenom, and two other California companies are offering similar cell-free prenatal tests. Ariosa was the first to sue, in 2011, seeking to prevent Sequenom from coming after it for patent infringement. An initial decision favored Ariosa, was appealed to a higher court, and came back to Illston’s court for a second review in light of recent Supreme Court decisions.

If Illston’s decision stands, the court ruling is a “big deal for the field” of DNA testing, says Henry Greely, an expert on law and bioethics at the Stanford University Center for Law and the Biosciences in Palo Alto, California. Limiting the reach of patent claims in this way will be good for public health, he argues. “This will help keep the prices down and provide a driver for general competition,” Greely predicts.

Some worry that the decision could scare potential investors away from the diagnostics industry. Ariosa’s Song doesn’t think so. A former venture capitalist, he says he’s confident investors can live with the rule changes. They won’t “give you a bunch of money because you have a patent on a protein or marker,” Song suggests. Instead, he says, they will have to vet the feasibility of business plans more carefully. Song acknowledges that he has patents, too, but insists they’re solid ones, based on “technology we developed ourselves.”

—ELIOT MARSHALL

PLANETARY SCIENCE

Orbiting MAVEN Mission Set to Trace A Planet's History in Thin Martian Air

Eons ago, planetary scientists believe, Mars was blanketed by a thick atmosphere that sheltered a surface awash with water—conditions that could have allowed life to emerge and thrive. Today, that atmosphere is thin and depleted, and the martian surface is a cold, barren desert. What caused this remarkable transformation?

If all goes well, a NASA spacecraft to be launched later this month will help answer that question. The Mars Atmosphere and Volatile EvolutioN (MAVEN) mission will fly through the outer fringes of Mars's atmosphere, measuring gases and monitoring conditions with eight instruments. The measurements should help researchers figure out how, over billions of years, the ceaseless wind of particles from the sun combined with asteroid impacts and Mars's own surface chemistry to gradually deplete its atmosphere of water vapor and other gases.

MAVEN is ultimately “about the habitability of Mars,” says Bruce Jakosky, the mission's principal investigator and a geologist at the University of Colorado, Boulder. “If you are trying to understand the potential for life on Mars, you have to understand its climate history.”

Until now, planetary scientists have attempted to answer the life question mainly from the planet's surface. While orbiters remotely mapped its topography and minerals, the three rovers that NASA has sent there over the past decade studied the ancient sediments for clues to water. That division of labor left out some important sources of information, says Chris Russell, a geophysicist at the University of California, Los Angeles, who is not on the MAVEN team. “We got into a certain trap of exploration—that we'll go down to the surface of Mars and we'll do it even better the next time,” Russell says. “We needed to break that mold, and MAVEN has done that.”

MAVEN will actually dive into the planet's thin upper atmosphere to sample it directly. Whereas previous Mars orbiters have flown between 257 and 400 kilometers above the surface, MAVEN will trace an elliptical orbit that will bring it 150 kilometers from martian soil, into the ionized outer reaches

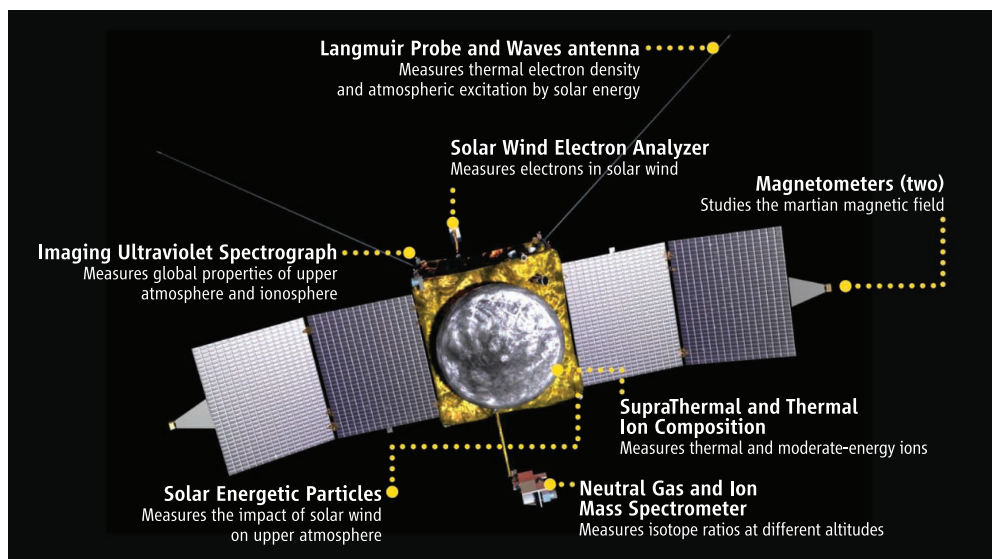
of the atmosphere. The spacecraft will also take five “deep dips” down to an altitude of 125 kilometers during its Earth-year-long rendezvous. That will allow its instruments to take data all the way from the ionosphere down to the edge of the lower atmosphere.

Six of MAVEN's instruments will study how the solar wind erodes the top of the martian upper atmosphere, which is not protected by the same kind of magnetic shield that fortifies Earth's atmosphere. The instruments include a magnetometer that will map Mars's magnetic field, which is believed to have been strong early in the planet's history but now exists only in magnetized patches of the mar-

researcher at NASA's Goddard Space Flight Center in Greenbelt, Maryland, who leads NGIMS. “This lets them escape the planet more easily.”

For example, Mahaffy says, measuring how the ratio of argon-38 to argon-36 changes with altitude would help researchers model the escape processes by revealing how quickly gases of different mass rise through the atmosphere. By combining those models with their knowledge of how the sun has changed over time, they could infer the density and composition of the martian atmosphere at different points in the planet's history.

Researchers think three processes could have thinned the atmosphere. “One is to take the carbon dioxide out of the atmosphere and form carbonate minerals in the crust,” Jakosky says, noting that spectral analysis of the martian surface by instruments on the



Mars bound. The MAVEN spacecraft, scheduled for launch later this month, will carry eight instruments to study the martian atmosphere in unprecedented detail.

tian crust. These pockets of magnetism shield parts of the atmosphere from the solar wind. The magnetometer's measurements, combined with another instrument's observations of the solar wind, will help researchers understand where the atmosphere is most vulnerable to being stripped away by the sun.

Another instrument, the Neutral Gas and Ion Mass Spectrometer (NGIMS), will measure the ratio of isotopes of elements such as argon in the upper atmosphere to give researchers a handle on the rate at which gases are escaping into space. “In the upper atmosphere, the gases are no longer fully mixed and separate out by mass, with the lighter gases able to move higher on average than the heavier gases,” says Paul Mahaffy, a

rovers provides evidence that this happened. Another possibility is that asteroids knocked off large amounts of gas; for this, too, there is evidence, in the form of impact craters seen on the planet. “The third process is escape to space,” driven by the solar wind. “Only by bringing together all of these processes can we begin to understand what has happened,” Jakosky says.

Along with its scientific payload, MAVEN will carry a DVD containing some 1100 haiku that the mission team received from the public in response to a contest announced last year. “Mars, your secret is / unknown for humanity,” reads one of the five winning entries. “We want to know you.”

—YUDHIJIT BHATTACHARJEE

SCIENCE AND THE MILITARY

Soldier-Scientists Join Counterinsurgency in Afghanistan

When Alexander Stewart went to war in Afghanistan, his foe was the unstable landscape. Every spring for a thousand years, an artificial reservoir on the Ghazni River has swollen with runoff as snowmelt gushes from the Hindu Kush mountain range. The current dam, the Band-e Sultan Dam, is so massive that “it’s something you can imagine the Tennessee Valley Authority might have built,” says Stewart, an assistant professor of geology at St. Lawrence University in Canton, New York. But the decades-old concrete edifice wasn’t built to last. On 29 March 2005, a section collapsed, sending a torrent downstream that flooded Ghazni city and drowned 14 people.

Four years after the disaster, Stewart was in Afghanistan poring over photos and reports for clues to why the Band-e Sultan gave out. Stewart, a glacier morphologist, was one of a dozen “soldier-scientists” assigned by the National Guard Bureau to an elite company, the U.S. Army’s 143rd Infantry Detachment, Long-Range Surveillance, for a 1-year tour of duty. Low-profile platoons like Stewart’s, deployed by the military’s Agribusiness Development Team (ADT) program, are part of the U.S.-led military coalition’s counterinsurgency strategy, which aims to coax Afghans to rely on their government rather than the Taliban.

It is the first time that soldier-scientists have been deployed on noncombat missions in a war zone, says Stewart, who described his experience at the Geological Society of America’s annual meeting in Denver last week. “It’s an unprecedented, modern approach to fighting,” he says.

Operating in 10 Afghan provinces (see map) for the past 5 years, the program has spent \$42 million on more than 680 projects, such as coring trees for climate records; shifting farmers from growing opium poppy to saffron; and assessing the Taliban’s potential to generate income from mining in their strongholds. Some geoscientists decry the effort. “They call it ‘weaponizing geology,’” says John Shroder, a geoscientist at the University of Nebraska, Omaha, whose research in Afghanistan predates the Soviet invasion in 1979. Shroder disagrees. “Scientists are doing a noble thing to help people there.”

Top brass are mulling whether to con-

tinue deploying such units after the coalition completes the drawdown of combat troops next year, according to the ADT Mission Office. And they may see action in future conflict zones: According to the National Guard’s 2012 Posture Statement, former U.S. Defense Secretary Robert Gates stated that the military is working to develop more programs like ADTs.

Before shipping out for Afghanistan, Stewart and the other scientist-soldiers were instructed how to build relationships with vil-

the Hindu Kush, you’re without water for 11 months,” he says. The Band-e Sultan’s stored water irrigates 15,000 hectares of cropland for about 25,000 families. The Afghan government had quickly mended the breach of the dam’s wing wall, but Stewart’s team wanted to figure out why the wall crumbled and assess the repair’s integrity.

The dam section that failed turned out to have been built on lake silt. “There was no structural stability,” Stewart says. And photos of the rubble revealed that the concrete lacked

reinforcement bars. Visiting Band-e Sultan that July, Stewart’s unease grew. He calculated that the “flimsy” new wing wall could reliably withstand the water pressure only if the reservoir was filled to less than 60% of the wall’s height. In the 4 years since Stewart’s assessment, the Afghan government has not followed through on promised sturdier repairs.

The Taliban, who regularly threaten to blow up the dam, have thwarted other missions, such as a plan to stock highland lakes with fish. A key dirt road leading into the mountains winds through Taliban territory. “We thought to airlift the [fish] tanks,” says Neal Litton, captain of the ADT that Stewart served on. “It was just too complicated.”

The expertise of scientists such as Stewart is vital, says Litton, now a financial adviser in Plano, Texas. “He’d tell me when something was not going to work.” But Litton says that other soldiers and superior officers didn’t always welcome such frank assessments from lower-ranking personnel—even if they are

Ph.D. scientists.

The satisfaction Stewart took in applying his expertise to helping Afghans was marred by tragedy. On 16 October 2009, when half of his company was returning from an ADT mission on a rugged dirt road from Jaghori, an improvised explosive device detonated beneath one of the armored Humvees, and mortars rained down. Christopher Staats, a renewable resources specialist, and Gabriel Green, a livestock specialist, died. “They were my brothers-in-arms,” says Stewart, who was at the base that day. A grim reminder that for soldier-scientists, danger is never far away.

—RICHARD STONE



Science in a combat zone. Geologist Alexander Stewart examines a repaired wall at the Band-e Sultan Dam in Ghazni, one of more than 680 missions.

lage elders, ascertain local needs, and send a proposal up the chain of command for funding. Among the missions of Stewart’s ADT was teaching villagers how to use wire gabion baskets to reinforce berms, showing farmers how to trellis grapes—“they had been growing them right on the ground,” Stewart says—and setting up an experimental farm for Ghazni University. It is vital work, Shroder says, because 3 decades of turmoil in Afghanistan “destroyed their education system.”

Stewart put his geoscience expertise to work on the dam collapse at Ghazni, which had serious repercussions for the region’s water and food security. “If you don’t control the 1 month of water you get from

PALEONTOLOGY

The Ears Have It: First Snakes Were Burrowers, Not Swimmers

LOS ANGELES, CALIFORNIA—Did the first snakes crawl on the land or swim in the water? It's one of paleontology's sharpest debates, pitting those who think that four-legged snake ancestors lost their limbs to become more efficient swimmers against those who believe that the demands of burrowing were key. The final verdict has yet to come in, but a new study of snake inner ears, presented at a paleontology conference* here, bolsters the view that today's snakes evolved from terrestrial, burrowing ancestors.

"We keep finding new evidence that keeps [the debate] alive," says paleontologist Thomas Holtz of the University of Maryland, College Park. Today, the majority of the world's 3500 species of snakes are terrestrial. But in 1997, paleontologist Michael Caldwell of the University of Alberta in Edmonton, Canada, and his colleagues reported the discovery of *Pachyrhachis*, a marine fossil snake that still had hind legs. They argued that the 100-million-year-old fossil pointed to extinct aquatic lizards—agile swimmers called mosasaurs—as the ancestors of snakes.

Other researchers demurred, and in 2006, some of those critics reported another transitional fossil: a 90-million-year-old burrowing snake named *Najash*—which retained

two legs, indicating it was primitive. In another strike against the aquatic hypothesis, some genetic studies indicated that snakes aren't closely related to living mosasaur relatives, such as the Komodo dragon.

Hong-yu Yi, a doctoral student with paleontologist Mark Norell at the American Museum of Natural History in New York City, thought she might find answers in an unexpected part of snakes' anatomy: their inner ears, which provide the sense of balance and equilibrium. Because aquatic snakes can orient and rotate their bodies in all directions in the water, she suspected that their inner ears would differ from those of terrestrial snakes. She CT-scanned 10 diverse modern snakes, aquatic and terrestrial, and, for comparison, nine modern lizards, finding that in aquatic snakes and lizards, the inner ear's semicircular canal projects relatively far from a disk-shaped ear structure called the vestibule. In modern burrowing snakes and terrestrial lizards, the semicircular canal projects much less and is partly fused with the vesti-

bule. Nonburrowing terrestrial snakes had an intermediate anatomy.

Snake fossils usually preserve the skull better than the sinuous body, so Yi looked at published scans of the inner ear of an 85-

million-year-old terrestrial snake called *Dinilysia*. Its structure clustered tightly with that of modern burrowers. "This new inner ear evidence adds support to the hypothesis that snakes have a terrestrial and burrowing origin," says evolutionary biologist Olivier Rieppel of the Field Museum in Chicago, Illinois.

But Caldwell argues that *Dinilysia*'s fossil ear lacks soft tissue and so can't be directly compared to that of living snakes; and that the 2-meter-long *Dinilysia* was much too big to be a burrowing snake, which today are much smaller. He also argues that today's burrowing snakes may have different adaptations to life underground—and different ear structures—than ancient burrowers.

Rieppel suggests Yi extend her study to include the smaller *Najash*. She might now have more specimens to analyze: Also at the meeting, *Najash*'s discoverer, Sebastián Apesteguía of Maimónides University in Buenos Aires, reported two new skulls of this ancestral snake.

—MICHAEL BALTER



By land not sea. New clues suggest that the earliest snakes, like 90-million-year-old *Najash*, shown in an artist's reconstruction, were terrestrial.

* 73rd Annual Meeting of the Society of Vertebrate Paleontology, Los Angeles, California, 30 October to 2 November.

Has Program to Rotate Scientists At NSF Spun Out of Control?

Anja Strømme still cries when she describes what happened to her this spring at the National Science Foundation (NSF). She hopes her story might help other scientists avoid the wrenching experience she went through.

The ionospheric physicist was finishing up her first year at the agency under a program that allows scientists to work for the federal government for a few years without severing their ties to their home institution. So-called rotators like Strømme are a vital cog in NSF's grantsmaking process. Roughly one in three NSF program officers is a rotator, as are a majority of the agency's senior managers.

Strømme managed the aeronomy program within NSF's Division of Atmospheric and Geospace Sciences. But on 14 March, the division director summoned her to his office. There, Strømme was told that she had committed "several federal offenses" relating to an alleged conflict of interest involving a grant proposal and that she "would be going to jail."

Leaving the meeting in a state of shock, Strømme spent the next few days trying frantically to find out more about her alleged misbehavior. But

she was never given the opportunity to defend herself, and within a week she was told that she no longer worked at the foundation. Strømme is now back in California at her previous employer, SRI International, which she says has been very supportive during her ordeal.

Online sciencemag.org

See special online reports (<http://scim.ag/NSFrotators>).

NSF officials credit rotators with keeping the agency on the cutting edge of science. But in recent years, some key members of Congress have raised questions about how NSF manages rotators, including their added cost and the loss of institutional memory. Those concerns have triggered three reports by NSF's in-house watchdog, the Office of Inspector General, leading to recommendations that NSF needs to do a better job of monitoring the program.

Strømme learned about another downside of the program in her last few days at NSF: Rotators aren't entitled to the same workplace protections afforded permanent employees. There is no mechanism for a rotator to respond directly to any allegations made against them, for example, nor to file a grievance.

Read more online about NSF's reliance on rotators, and why they are so vulnerable if tensions arise in the workplace.

—JEFFREY MERVIS



The Forgotten Malaria

Long considered “benign,” the malaria parasite *Plasmodium vivax* threatens billions while eluding control measures. Now, scientists are going on the offensive

IT HAS PLAGUED ENGLISH KINGS AND AS many as eight U.S. presidents—including George Washington and Abraham Lincoln—and may have helped kill Genghis Khan. It causes wracking fever and chills, headaches and muscle pain, and can lead to severe anemia. After you think you have beaten it, it can return again, months or even years later, starting the same cycle of misery. Roughly one-third of the world’s population is thought to be at risk, mostly in Asia and Latin America. And almost no one has ever heard of it.

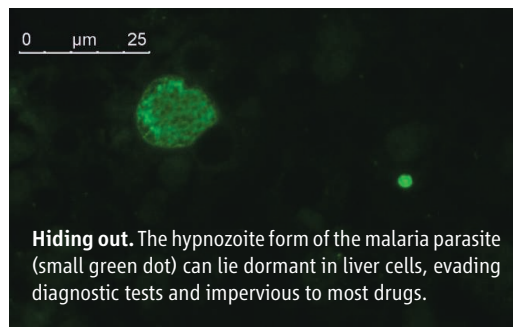
It is *Plasmodium vivax*, one of the five *Plasmodium* species that infect humans and cause malaria (see table, p. 685). For many years, vivax malaria was officially called “benign” malaria, in contrast to the “malignant” variety caused by its cousin *Plasmodium falciparum*. Falciparum malaria is undeniably deadly, killing more than 650,000 people each year—mostly children in sub-Saharan Africa—and accounting for more than 90% of global malaria deaths. Because it is so lethal, it has received

most of the attention—and funding—in the global fight against the disease.

In contrast, vivax malaria has long been an afterthought in both public health plans and in research funding. But that is beginning to change, in part because the global campaign against malaria is starting to pay off. Since the wide-scale introduction of bed nets and new drugs, malaria rates have fallen dramatically across the globe. There is even talk about eventually eradicating the disease (*Science*, 7 December 2007, p. 1544). But vivax malaria can evade some of the

standard malaria-fighting tools, so as overall rates of malaria fall in a region, the proportion of cases that are caused by *P. vivax* increases. And although vivax malaria kills fewer people, it is decidedly not benign. “The saying used to go: Vivax malaria doesn’t kill you, but you feel like it will,” says Robert Newman, director of the Global Malaria Programme at the World Health Organization (WHO) in Geneva, Switzerland.

Now, as public health leaders intensify their push to eliminate the disease from more and more regions, they are also starting to give *P. vivax* special attention. For the first time, Newman says, WHO is drawing up a specific plan to address vivax malaria as part of its updated global malaria-fighting strategy, due out in 2015. The plan is likely to include, for example, better diagnosis of parasite species in patients and updated treatment guidelines. “We have recognized the deficiencies of always making vivax an afterthought,” he says. The fight against vivax may also bene-



Hiding out. The hypnozoite form of the malaria parasite (small green dot) can lie dormant in liver cells, evading diagnostic tests and impervious to most drugs.

CREDITS (TOP TO BOTTOM): MEDICALRECOM/VISUALS UNLIMITED INC.; ANNE-MARIE ZEEMAN

Downloaded from www.sciencemag.org on November 7, 2013

Feverish attacks. *Plasmodium* parasites (yellow in this artist's rendition) infect and kill red blood cells, causing symptoms of malaria.

fit from a new drug to overcome the parasite's best defense: its ability to lie dormant in the human liver.

Richard Feachem, the director of the Global Health Group at the University of California, San Francisco, who has long warned about the threat of vivax malaria, argues that such strategies will be crucial to defeating malaria. "The final battle against malaria is a battle against vivax."

A formidable foe

In several ways, *Plasmodium vivax* is even more exquisitely adapted to its human host than *P. falciparum* is. Unlike *P. falciparum*, *P. vivax* can populate the bloodstream with sexual-stage parasites—the form picked up by mosquitoes on their way to the next victim—even before a patient shows symptoms. That means treating symptomatic patients promptly doesn't necessarily help stop an outbreak, as it does with falciparum malaria, in which fevers occur at the same time as sexual stages develop. When the debilitating symptoms do set in, they make the human host miserable but are usually not fatal—at least in the short term—which allows the parasite to continue multiplying. And when vivax hides out in the liver, it causes no symptoms and is undetectable in blood tests, only to reappear weeks or even many months later, once again tormenting the human host and ready, again, to infect new mosquitoes.

"Falciparum kills you quickly. Vivax kills you slowly," says malaria researcher Ric Price of the University of Oxford in the United Kingdom and the Menzies School of Health Research in Darwin, Australia. At first glance, clinical cases of both vivax and falciparum malaria look similar. Both cause the classic symptoms of alternating fever, chills, and sweats. Both kinds of malaria invade the blood. But whereas *P. falciparum* attacks red blood cells of all ages, *P. vivax* targets reticulocytes, immature red blood cells that are relatively rare, which can make vivax infections hard to diagnose. When *P. falciparum* invades red blood cells, it makes them rigid, which is thought to lead to blocked capillaries,

a factor in the deadly cerebral malaria. Blood cells infected with *P. vivax* seem to remain pliable, which might explain why it isn't quite as lethal.

How and why *P. vivax* causes severe malaria is unclear, but recurrent episodes leave people chronically anemic—children are especially vulnerable, Price notes. A single infectious bite can trigger six or more relapses a year, as the dormant parasites in the liver activate and cause a new bout of disease. Fighting off those episodes can leave people more vulnerable to other diseases—and to poverty and malnutrition, because they can't work while ill. Other infectious diseases,

of different strains and the clinical course of the disease. Vivax malaria "is one of the only diseases or infectious agents that we've forgotten more about than we've learned in recent years," says Nick White, a professor of tropical medicine at Mahidol University in Bangkok and the University of Oxford.

"We have a vivax research deficit," Feachem says. Although scientists discovered in the 1970s how to grow *P. falciparum* in the lab, there is still no system for culturing *P. vivax* for more than a few weeks. It has even been unclear just how many people *P. vivax* sickens and where the toll is the highest. Malaria cases are notoriously hard

Malaria Parasites That Infect Humans

Species	Global distribution*	Distinguishing characteristics	Dormant liver stage?
<i>Plasmodium falciparum</i>	80–90% of cases in Africa; 40–50% of cases in Western Pacific and Southeast Asia; 4–30% in South Asia, South America, and rest of tropics	Causes a majority of deaths globally; most prevalent species in sub-Saharan Africa	No
<i>Plasmodium vivax</i>	70–90% of cases in most of Asia and South America, 50–60% of cases in Southeast Asia and Western Pacific, 1–10% in Africa	Duffy antigen, common in African populations, provides immunity to <i>P. vivax</i> ; can cause severe malaria in roughly 1/5 of cases	Yes, up to 2 years dormancy
<i>Plasmodium malariae</i>	2–3% in Africa, sporadic in Asia and South America	Produces fever at 3-day intervals instead of typical 2-day; clinically milder symptoms; little-studied, disease burden probably underestimated	No
<i>Plasmodium ovale</i>	8% of cases in parts of Africa, stray cases in Asia	Clinically similar to <i>P. vivax</i> , though with milder symptoms; disease burden probably underestimated. May be two distinct species	Yes, up to 4 years dormancy
<i>Plasmodium knowlesi</i>	Reported from Southeast Asia; 70% of cases in some of those areas	Primarily infects macaques; becoming more prevalent in humans	No

*Percentages are rough estimates because specific species are rarely diagnosed in patients

including falciparum malaria, also exacerbate the disease because they seem to trigger relapses. "It's a cofactor in a lot of people's deaths," says Simon Hay, an epidemiologist at the University of Oxford.

As researchers delve into vivax malaria, it is becoming apparent just how little is known about the parasite. Much of the research on it was done more than half a century ago, when the parasite was endemic across North America and northern Europe. (Tens of thousands of people were infected in outbreaks across the Netherlands and northern Germany after World War II.) In a strange twist, *P. vivax* was even used from the 1920s until the 1940s as a radical treatment for end-stage syphilis (see sidebar, p. 686). The "malariotherapy" practitioners recorded and published clinical data on hundreds of patients, documenting the behav-

ior to count, because they occur overwhelmingly in regions where health care—and health care records—are lacking (*Science*, 15 June 2012, p. 1372). And not all diagnostic tests distinguish whether a patient is suffering from vivax or falciparum malaria.

To help fill the gap, the Malaria Atlas Project, which Hay coordinates, has used geographic, population, and epidemiological information to estimate the number of people at risk of contracting *P. vivax* (*Science*, 6 August 2010, p. 618). Their latest analysis concluded that 2.5 billion people are at risk, which means they are living in places where the parasite has been reported and where mosquitoes can transmit it.

Most of those are in Asia and Latin America. Although *P. vivax* used to be entrenched in Europe and the United States, it disap-

peared as countries became wealthier and housing conditions improved. (One of the most effective, though expensive, antimalaria measures is mosquito-proof housing with screens on the windows and doors.) Now, it is a plague of low- and middle-income countries, except those in sub-Saharan Africa, where the *P. vivax* map has a conspicuous hole. There, most of the population lacks the Duffy antigen, a protein on the surface of red blood cells that *P. vivax* uses

to invade the cells. The parasite can't spread effectively in populations in which most people are "Duffy-negative."

P. vivax is carried by at least 71 species of mosquitoes, which gives it another edge over *P. falciparum*. Many vivax vectors live happily in temperate climates—as far north as Finland. Some even prefer to bite outdoors or during the daytime, so in some regions indoor insecticide spraying and bed nets are less effective at preventing the dis-

ease. Several key vector species haven't ever been grown in the lab for closer study, and it's also unclear how widespread insecticide resistance might be.

One bright spot is that, like falciparum malaria, vivax malaria is treatable. In fact, *P. vivax* has so far developed less resistance to common drugs. Chloroquine, which has lost most of its potency against *P. falciparum*, can still effectively treat acute infections of *P. vivax* in most regions.

But those treatments are powerless against the parasite's nastiest trick: its ability to lurk in the liver, making it much harder to cure than *P. falciparum*. Both parasites, when they are transmitted from mosquito to human host, go first to the liver. But unlike *P. falciparum* parasites, which undergo asexual reproduction in the liver and then burst out to infect red blood cells, some vivax parasites simply go into hiding. They form what are called hypnozoites (the name derives from "sleeping parasites"), an especially small form that nestles inside a liver cell. There they remain for weeks, months, or even years, waiting until chances are good that a recipient mosquito will be available to pass sexual stages on to the next victim. In fact, it is the hypnozoites that allow the parasite to survive in more temperate zones, where mosquitoes bite only part of the year. The parasite can bide its time for 9 months or even longer—enough time to wait out the winter and allow new vectors to hatch. (Another parasite, *Plasmodium ovale*, can also form hypnozoites, but it is much rarer than *P. vivax*.)

Hypnozoites can't be detected by blood tests, so the best strategy to eliminate the parasite from a region would be to mass-treat populations with a drug that could kill the sleeping pathogens. One drug can do that: Called primaquine, it was developed in the 1940s and early 1950s. It has two major drawbacks, however. The standard dose requires people to take a daily pill for 14 days—not an easy thing to enforce in people who don't feel ill. And in people who have a genetic condition called G6PD deficiency, the drug causes red blood cells to burst open. In severe cases, the reaction, called hemolysis, can be fatal.

G6PD stands for glucose-6-phosphate dehydrogenase, an enzyme that is especially important in red blood cell metabolism. There is no cheap, easy field test for G6PD traits, so any mass treatment campaign risks severe side effects in some people. The variant also seems to confer some protection against vivax malaria, so that regions in which the parasite is common tend to have more people with the trait.

Malaria as Lifesaving Therapy

Vivax malaria was once familiar to doctors not only as a foe, but also as an ally. In the first half of the 20th century, people with tertiary syphilis, the phase of the disease in which *Treponema pallidum* bacteria attack the brain and nervous system, were doomed to a gruesome death: They would become increasingly neurotic and gradually paralyzed. Most were institutionalized, and there was no cure.

So physicians drew on a theory that had gained prominence in the late 19th century—that high fever could help cure a variety of mental illnesses. Austrian psychiatrist Julius Wagner-Jauregg was one of the idea's strongest proponents, but his initial experiments in the 1880s with fever-inducing pathogens and compounds such as tuberculin and salmonella toxin failed. He reasoned that the fevers they induced were not severe enough. In 1917, he tried again when a soldier who had caught malaria while fighting in the Balkans was admitted to his ward. Wagner-Jauregg used the soldier's blood to inoculate nine neurosyphilis patients. Six of them got better.

Soon, malariotherapy was seen as a miracle cure. It became a leading treatment for end-stage syphilis, quickly spreading across Europe and North America. Between 1917 and the rise of penicillin in the 1940s, tens of thousands of syphilis patients were infected with malaria. Different clinics used different parasites, but "after a few costly mistakes" with *Plasmodium falciparum*, a more lethal parasite, "most people settled on vivax," says Nick White, professor of tropical medicine at Mahidol University in Bangkok and the University of Oxford in the United Kingdom.

No one is sure exactly how it worked, but the high fevers that resulted seemed to help the patients' immune systems fight off the bacteria. Roughly half recovered enough to resume normal activities; many were able to resume independent lives. In 1927, Wagner-Jauregg won the Nobel Prize in physiology or medicine for his discovery. (The Austrian's legacy has a darker side as well. He was an advocate of eugenics, and in the 1930s he was a Nazi supporter.)

Because of malariotherapy, "the parasite's biology was investigated in amazing detail," White says. The result was a wealth of information about the disease process and how it differs depending on the strain of *P. vivax*. Those records are gaining some new attention today, as vivax starts to emerge from the shadow of its better-known cousin, falciparum (see main text, p. 684).

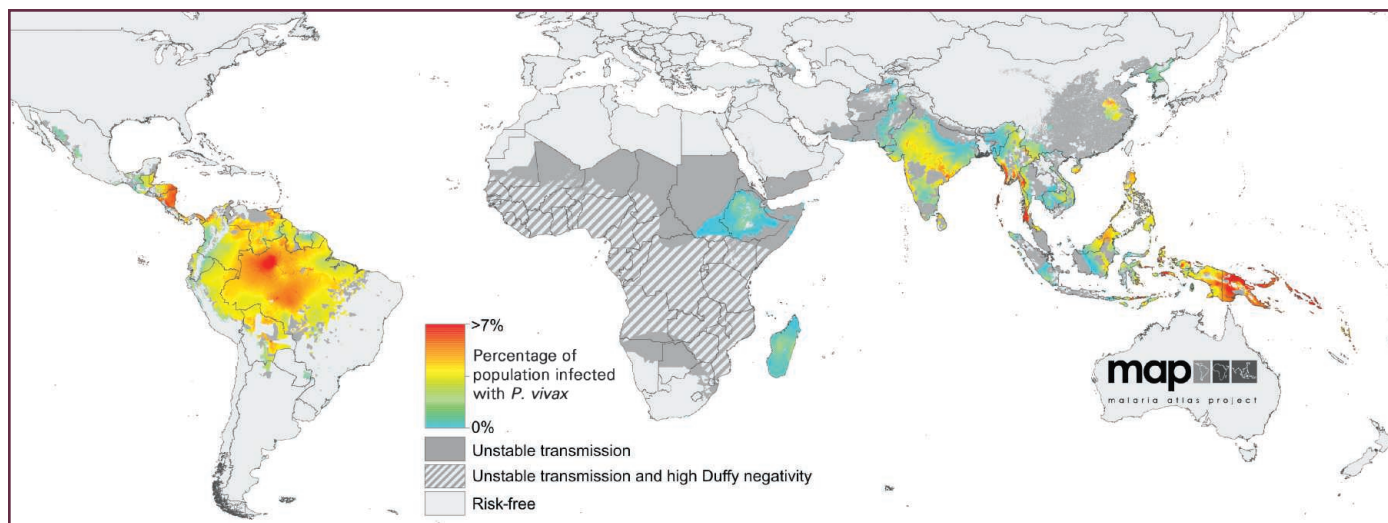
But this medicinal use of the parasite is in part to blame for the later neglect of the disease it causes, says Kevin Baird of the Eijkman Oxford Clinical Research Unit in Jakarta. Because doctors used *P. vivax* as a medical treatment, he says, many people assumed that it must be relatively harmless. But Baird says that even at the height of the therapy's use, that reputation was unfounded: Although malariotherapy saved lives, it also killed as many as 15% of the patients who received it, despite close monitoring and treatment of the infection.

—G. V.



Radical treatment. In a 1934 image, Julius Wagner-Jauregg (center, in black suit) watches as blood from a malaria patient (in background on left) is injected into a neurosyphilis patient (center).

Prevalence of *Plasmodium vivax* Malaria in 2010



Widespread threat. The Malaria Atlas Project estimates that 2.5 billion people are at risk for vivax malaria. The parasite spares many Africans because they lack a blood protein called the Duffy antigen.

Another complication is that the drug itself is not well understood. No one knows how the body processes it or how it attacks the hypnozoite. The standard 2-week dose stems from the 14 days it took to ship malaria-infected soldiers home from the Korean War. Studies have shown that people treated with the drug have fewer relapses, but there is little definitive data on dosage, efficacy, or resistance.

Hopeful glimmers

An improved, if imperfect, alternative is raising hopes. At the annual meeting of the American Society of Tropical Medicine and Hygiene in Washington, D.C., next week, researchers are scheduled to present results from a long-awaited trial of what could be the first new drug to target liver-stage parasites in half a century, tafenoquine. Its main advantage: A single dose, instead of a 14-day course, seems to be sufficient. That “would be a game-changer,” Newman says.

Tafenoquine was developed by researchers at the Walter Reed Army Institute of Research in the 1970s and tested, decades ago, in safety trials in thousands of healthy volunteers. It languished, however, until the push for malaria elimination sparked new interest in alternatives for primaquine, says Tim Wells, chief scientific officer of the Geneva-based nonprofit Medicines for Malaria Venture (MMV), which is helping coordinate the trial with the pharmaceutical company GlaxoSmithKline (GSK). In 2011, the partners launched the trial testing the effectiveness of a single dose of tafeno-

quine compared to a standard course of primaquine in 320 malaria patients in Brazil, India, Thailand, and Peru. (The drugs were combined with a standard 3-day course of chloroquine to treat blood-stage parasites.)

Although GSK scientists can’t talk about the study before the meeting, initial trial results posted on the company website indicate that 90% of patients who received a single 300- or 600-mg dose of the drug were relapse-free 6 months later. Among patients who received primaquine, 24% relapsed within 6 months. “The data are absolutely spectacular,” Wells says. Ideally, he says, researchers will be able to combine the safety data from the Army’s earlier trials with the new study in a submission to the U.S. Food and Drug Administration for approval.

Tafenoquine is chemically similar to primaquine, so it still causes hemolysis in people who are G6PD deficient. Wells says GSK will apply for its approval only in combination with a test for G6PD deficiency. That could limit its use in some regions. Still, Newman says, a single dose is a huge improvement over a 14-day course of drugs. If the results hold up, he says, “tafenoquine will fix a lot of things.”

Although other antihypnozoite drug candidates are sparse, the search for alternatives is gaining momentum. In 2008, the first *P. vivax* genome was sequenced. Since then, several more strains have been added to the databases, allowing researchers to identify new potential weak spots. Primate models

and new ways to grow the parasite in vitro are also helping researchers search for new compounds that can target the parasite’s liver stages.

Sangeeta Bhatia, a bioengineer at the Massachusetts Institute of Technology in Cambridge, has developed a way to grow human “microivers,” liver cells growing together with supporting cells that can live in the lab for 4 to 6 weeks. In July, Bhatia and her colleagues described in *Cell Host & Microbe* how they were able to grow liver stages of both *P. falciparum* and *P. vivax*. The researchers saw miniature forms of the parasite in some of their vivax cultures, which may be hypnozoites. The scientists did not see any of the small forms reactivate, Bhatia says, so they haven’t yet proven that they have cultured hypnozoites, but she and others think it is likely. “We have a glimpse of the organism for the first time in vitro,” she says.

Despite the challenges, Feachem says he is optimistic that with new attention to *P. vivax*, the parasite can ultimately be defeated, even in tropical regions where it seems hardest to tackle. He points to Taiwan, Singapore, and the Maldives as places that, with sufficient investment, managed to eliminate the parasite with existing tools.

A century ago, malaria “was a global disease. It was endemic in every country ... including regions north of the Arctic Circle,” Feachem says. More than 100 countries have eliminated malaria in the past century, he says; all of them overcame vivax. To be sure, he says, many of those victories were closely linked to economic development. Still, he says, “we’ve won this war 100 times in the past 100 years. It’s not like we can’t do it.”

—GRETCHEN VOGEL

Online

sciencemag.org

Podcast interview with author Gretchen Vogel (http://scim.ag/pod_6159).



CLIMATE CHANGE

In the Hot Seat

Scientists pressed on global warming's link to weather disasters are scrambling to grasp the teachable moment without going beyond their meager understanding

Many climate scientists winced earlier this year when a well-meaning nonscientist tried to use extreme weather to argue that global warming is real. "We can choose to believe that Superstorm Sandy, and the most severe drought in decades, and the worst wildfires some states have ever seen were all just a freak coincidence. Or we can choose to believe in the overwhelming judgment of science—and act before it's too late."

That was President Barack Obama in his State of the Union address. The fact is, there is little or no evidence that global warming steered Sandy into New Jersey or made the storm any stronger. And scientists haven't even tried yet to link climate change with particular fires.

Representative Lamar Smith (R-TX), one of the president's political opponents, got it just as wrong in a recent newspaper editorial titled "Extreme weather isn't linked to climate change." In fact, it is, sometimes. Climate models have securely linked several heat waves to global warming, which can increase the odds of extreme heat many fold.

For climate scientists, extreme weather is risky territory. There

is no question that global warming is real, but the science linking any one hurricane, drought, or flood to climate change is shaky, at best. And yet politicians, the public, and the rare scientist inevitably seize on vivid, easy-to-grasp weather events to make their points about abstract, long-term climate. Add in the loud voices of climate activists like Al Gore and 350.org's Bill McKibben, and the climate change discourse is "as much politics as scientific evidence," says climate scientist Martin Hoerling of



Ha, ha. Grandchildren of global-warming skeptic Senator James Inhofe and their parents built a mocking igloo after a 2010 snowstorm battered Washington, D.C.

Where's the science?

For better or worse, extreme weather is persuading Americans to take global warming seriously. In March and September 2012, for example, climate and media researcher Anthony Leiserowitz of Yale University with his Yale and George Mason University colleagues surveyed more than 1000 Americans (<http://tinyurl.com/l697t4o>). In September, 74% of those polled agreed that "global warming is affecting weather in the United States." That was up by 5% from March, after a summer of record drought, high temperatures, and powerful storms. And substantial majorities

said global warming had worsened every one of six recent extreme weather events in the United States—from high temperatures to forest fires to a blustery "derecho."

The public perception is that "we live on a new planet of extreme weather," notes communication researcher Matthew Nisbet of American University in Washington, D.C. "That's a very engaging narrative."

But it is one that makes many mainstream climate scientists uneasy. As summarized in a full-page table in the IPCC report, their confidence linking the observed global warming to extreme weather does not

Burning question. It is plausible that global warming made recent Australian bushfires more likely, but researchers can't say for sure.

the National Oceanic and Atmospheric Administration (NOAA) in Boulder, Colorado.

But climate researchers aren't giving up on turning extreme weather events into moments of teachable science. September's mammoth international assessment from the Intergovernmental Panel on Climate Change (IPCC) (*Science*, 4 October, p. 23) details current understanding—such as it is—of extreme weather's links to climate change, and describes new methods for gauging those links. With more effort, climate scientists could one day answer the "Is this climate change?" question on the spot.

CREDITS (TOP TO BOTTOM): DANIEL MUNOZ/REUTERS/NEWSCOM; TOM WILLIAMS/ROLL CALL PHOTOS/NEWSCOM

Downloaded from www.sciencemag.org on November 7, 2013

extend much further. There “very likely” have been fewer cold and more frequent hot days and nights since 1950, the report concludes, and humans “very likely” contributed to the changes. In a physically straightforward next step, the report finds that heat waves have become more frequent, longer, or both. And heavy precipitation events have become more frequent because warmer air can hold and then release more moisture.

But when it comes to the role of human-induced change in phenomena that are more than one step removed from a simple warming, the IPCC typically has “low confidence.” That goes for droughts (down from medium confidence in the 2007 assessment), floods, and tropical storms. Wildfires aren’t even considered, for good cause. It’s plausible that a warming climate plays a role in fires in places like the western United States and Australia, says wildfire researcher Max Moritz of the University of California, Berkeley. But “fire is a couple steps removed from temperature or precipitation, and our records are short. So detecting a trend is tough and attributing an event to climate change is really, really tough. We have to be very careful.”

Wrong messengers

Links between extreme weather and climate change are not only often scientifically suspect, they may also be a risky strategy for persuading the public to take climate change seriously. “What disturbs me is assigning anything that comes along to global warming,” says professor emeritus of meteorology John M. Wallace of the University of Washington, Seattle. “That may work in the short run, but I don’t think that kind of conversion has staying power.” Indeed, surveys coming out on the 1-year anniversary of Hurricane Sandy’s landfall (29 October) show the concerns about hurricanes that spiked in the wake of the disaster have nearly faded away.

And “there’s a little bit of ‘live by the sword, die by the sword’” in making the connection, Leiserowitz says. A steady stream of extreme weather events makes for a steady media drumbeat on climate change, but that stream can falter. The current Atlantic hurricane season looks to be a near no-show with just two short-lived, minimal hurricanes

(Category 1) so far with a month to go in the season and nothing stirring in the tropical Atlantic. And no major hurricane (Category 3 to 5) has struck the U.S. coast since 2005. (Sandy may have been a “super” storm, but it wasn’t a major hurricane.)

When the weather turns cold, it becomes a cudgel for climate skeptics. The “Snowmageddon” that hit Washington, D.C., in February 2010 with 70 centimeters of snow brought out a less-than-playful taunt from conservative U.S. Senator James Inhofe. His grandchildren

Try, try again

As long as reporters and the public insist on blurring climate change and run-of-the-mill weather, however, experts must manage as best they can. At the nonprofit Climate Central in Princeton, New Jersey, scientists and journalists work with the public’s go-to people on weather: local TV meteorologists. “In these moments [of extreme weather], people have questions, we provide the context,” says Climate Central’s chief climatologist, Heidi Cullen. “Sometimes it’s a little bit messy, but we try to be really, really careful. We educate people about the scientific method itself.”

Climate scientists are also working on developing better talking points. “I talk about the risk,” says climate scientist Peter Stott of the U.K. Met Office’s Hadley Centre in Exeter. “Sometimes it has been quite successful. People understand there’s always been extreme weather.” By consulting climate records and modeling extreme events with and without added greenhouse gases, scientists can talk about how much global warming has increased the chances of extreme events—without blaming any one event on warming. For example, a NOAA and U.K. Met Office study published in the July 2012 *Bulletin of the American Meteorological Society* found that heat waves like the one that scorched Texas in 2011 are now 20 times as likely to occur as they were 50 years ago given the same conditions in the tropical Pacific that favor them.

That message came a year after the heat waves—fast for a meteorological study, but too slow to influence public perceptions. But Myles Allen thinks modelers can forge links between global warming and particular extreme weather much faster. Allen, a climate scientist at the University of Oxford in the United Kingdom, says models used to forecast the next season’s climate could be adapted to calculate the probability of a range of extreme events. “It wouldn’t be all that difficult, though it would require substantial funding,” Allen says, “but we should do it.” Then, when the first reporter calls in the midst of the next heat wave, there might be a firmer answer to that nagging perennial question.

—RICHARD A. KERR

Global warming had a role here ...



... But not here



It depends. Global warming boosted the chances for heat and dryness in Texas in 2011 (*top*), according to published studies, but it had nothing to do with upping the 2011 rains in Thailand (*bottom*).

and their parents built a much-photographed igloo with a sign reading “Al Gore’s New Home.” Actually, Inhofe’s taunt was baseless; global warming favors heavier precipitation, both wet and white.

Indeed, the whole field of extreme weather is a minefield for scientists. “Extreme events are the last place you want to look to document the human effect” of climate change, notes science policy scholar Roger Pielke Jr., of the University of Colorado, Boulder. Uncertainties and unknowns are so abundant in the field that “I’ve advocated for a long time that extreme events should not be part of the public dialogue,” he says.

LETTERS

edited by Jennifer Sills

In Defense of WHO's Blood Donation Policy

THE WORLD HEALTH ORGANIZATION (WHO) HAS LONG RECOMMENDED VOLUNTARY NON-remunerated blood donation (VNRBD) as the foundation for safe, reliable, and adequate blood supplies. In their Policy Forum "Economic rewards to motivate blood donations" (24 May, p. 927), N. Lacetera *et al.* argue that "the most relevant empirical evidence shows positive effects of offering economic rewards on donations" and suggest that WHO should consider changing its policy. On behalf of the Health Systems and Innovation Cluster of WHO, I disagree.

Lacetera *et al.* do not distinguish between unacceptable economic rewards for blood donation (such as US\$15 or \$25 supermarket vouchers) and acceptable small tokens (such as a free cholesterol test). The WHO VNRBD policy permits the use of small tokens of appreciation for blood donors (1, 2), a stance consistent with the Nuffield Council on Bioethics's "Intervention Ladder," a useful tool for analyzing the ethical acceptability of different forms

of encouragement for donating bodily material in various circumstances (3). Folléa *et al.* compares each of the six "rungs" of this "Intervention Ladder" with the definition of VNRBD of the Council of Europe (1, 4). The Oviedo Convention (5), a binding international legal instrument developed by the Council of Europe, is also consistent with this policy.

Lacetera *et al.* argue that the WHO position is based on uncontrolled studies and nonrandom samples. The use of evidence in public health decision-making is more complex than in clinical practice (6) and needs to draw on sources beyond the traditional hierarchy of study designs while addressing equity, transferability, acceptability, patient preferences, and social values. Reliance on evidence of different grades of robustness rather than exclusively on randomized controlled trials (RCTs) is not unusual for health policy studies. An overemphasis on RCTs poses important ethical and logistic problems and may incur avoidable deaths, particularly in resource-poor settings (7).

A change in VNRBD policy would require evidence across a range of contexts in different settings that addressed safety, donor recruitment, impact on social cohesion and solidarity, effect on concomitant VNRBD

programs, and avoidance of the exploitation of the poor and vulnerable, as well as the assessment of potential negative health and social side effects on a large scale.

VNRBD leads to a safer blood supply. Evidence shows significantly lower prevalence of transfusion-transmissible infections among voluntary nonremunerated donors than among other types of donors (8–10). Volkow *et al.* have shown that injection drug users from two Mexican-U.S. border cities rarely donate in Mexico, where payment for donations is banned, but do so across the border in the United States, where payment is allowed (11). These donors tend to deny their risk behavior, putting the blood supply at risk. The U.S. General Accounting Office Testimony of 1997 showed that test-positive rates for commercial plasma donors were 2 to 20 times higher than those for volunteer whole blood donors across a range of tests (12).

VNRBD also improves the supply of blood (8–10, 13). In a review of self-reported motivators and deterrents for blood donation, donors indicated that monetary incentives were unwanted, whereas nondonors indicated that these would be inadequate to motivate them to donate (14). Abolghasemi *et al.* reviewed over 20 epidemiological, economic, and psychological studies from the past four decades and concluded that offering money or cash-equivalent incentives may have negative effects on both blood safety and blood donor contributions (15). When systems of paid and voluntary blood donation coexist, people who might otherwise donate voluntarily may opt to receive payment for their blood, crowding out voluntary blood donor programs (8).

Lacetera *et al.* ignore an important consideration underlying VNRBD: donor protection. By providing underprivileged populations in need of money with financial incentives to donate, the commercial collection of blood, plasma, and cellular blood components could exploit the poor and vulnerable, in opposition to the directives of the Universal Declaration on Bioethics and Human Rights (16).

Numerous examples from both developed and developing countries, including Sri Lanka and Kenya (17, 18), show that VNRBD can provide a strong foundation for sustainable blood systems. Research in this field should focus on how to protect and further strengthen VNRBD programs.

NEELAM DHINGRA

Blood Transfusion Safety (on behalf of WHO, Health Systems and Innovation Cluster), World Health Organization, Geneva, 1211, Switzerland. E-mail: dthingran@who.int

References

1. Council of Europe Committee of Ministers, "Recommendation No. R(95)14 of the Committee of Ministers to member states on the preparation, use and quality assurance of blood components"; adopted by the Committee of Ministers on 12 October 1995, at the 545th meeting of the Ministers' Deputies (www.edqm.eu/site/Recommendation_No_R9515_on_the_preparation_use_and_quality_assurance_of_blood_components-en-4019-2.html).
2. *Towards 100% Voluntary Blood Donation: A Global*

- Framework For Action (World Health Organization and International Federation of Red Cross and Red Crescent Societies, Geneva, 2010); www.who.int/bloodsafety/publications/9789241599696/en/index.html.
3. *Human Bodies: Donation for Medicine and Research* (Nuffield Council on Bioethics, London, 2011); www.nuffieldbioethics.org/sites/default/files/Donation_full_report.pdf.
 4. G. Folléa, E. Seifried, J. de Wit, *Blood Transfus.* 10.2450/0011-13 (2013).
 5. Convention for the Protection of Human Rights and Dignity of the Human Being with Regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine (Oviedo Convention, Council of Europe, Oviedo, Spain, 1997); <http://conventions.coe.int/Treaty/en/Treaties/Html/164.htm>.
 6. L. Orton *et al.*, *PLOS ONE* 6, e21704 (2011).
 7. M. Potts *et al.*, *Br. Med. J.* 333, 701 (2006).
 8. T. Eastlund, *Transfusion* 38, 874 (1998).
 9. C. L. van der Poel, E. Seifried, W. P. Schaasberg, *Vox Sang.* 83, 285 (2002).
 10. V. Kalibatas, *Vox Sang.* 94, 209 (2008).
 11. P. Volkow *et al.*, *Int. J. Drug Pol.* 20, 409 (2009).
 12. U.S. General Accounting Office Testimony, "Blood safety: Enhancing safeguards would strengthen the nation's blood supply" (GAO/T-HEHS-97-143, 1997); www.gao.gov/archive/1997/he97143t.pdf.
 13. J. R. Cruz, *Lancet* 365, 1463 (2005).
 14. T. C. Bednall, L. L. Bove, *Transfus. Med. Rev.* 25, 317 (2011).
 15. H. Abolghasemi, N. S. Hosseini-Divkhalayi, F. Seighali, *Asian J. Transfus. Sci.* 4, 9 (2010).
 16. *Universal Declaration on Bioethics and Human Rights* (United Nations Educational, Scientific, and Cultural Organization, Paris, 2005); http://portal.unesco.org/en/ev.php-URL_ID=31058&URL_DO=DO_TOPIC&URL_SECTION=201.html.
 17. "Self-sufficiency in blood supply based on voluntary unpaid donors: an achievable goal" (2013); www.who.int/features/2013/world_blood_donor_day/en/.
 18. S. V. Basavaraju *et al.*, *Vox Sang.* 99, 212 (2010).

Response

DHINGRA STRESSES THE DIFFERENCE BETWEEN rewards and tokens of appreciation for blood donations. He points to the Nuffield Council guidelines, which explicitly warn that any benefits that "encourage those who would not otherwise have contemplated donating to consider doing so" should be scrutinized because they might be harmful [(1), page 7]. The incentives in the studies that we reviewed are deemed consistent with the ethical and professional standards of the blood banks offering them. These studies show that economic rewards can motivate people to make donations that would not have occurred otherwise, without negative consequences on safety. Our conclusion thus

stands: Existing guidelines should be reconsidered to recognize a role for incentives in generating additional, safe donations.

Randomized controlled trials (RCTs) are not the only form of evidence that should inform policy. However, when available, RCTs are the current best practice. Equally important, it is not merely the RCT nature of the studies that we reviewed that makes them compelling; it is also critical that evidence is based on actual behavior of people in response to actual incentive offers, and on large representative samples. For socially desirable activities, relying on self-reported motivations or laboratory settings can be misleading. Dinghra's example highlights this concern; a free cholesterol test favored by respondents in surveys had no effect when actually offered to blood donors in the field, whereas a hypothetical lottery ticket not favored by respondents increased donations when actually offered (2, 3). The remarkable aspect of the reviewed evidence (both RCT and observational) is the consistency of findings despite the different contexts (United States, Italy, Switzerland, and Argentina), types of items offered, and types of data.

Uncontrolled studies that do not meet these standards—such as most of those reviewed in van der Poel *et al.* (4) as well as the others that Dinghra cites—should be taken with great caution (5). Indeed, there is no existing evidence meeting the highest current standards to support the claim that voluntary donation increases safety and supply, and none of the studies that Dinghra cites causally identify the effects of unconditional economic incentives on safety on representative samples and for actual blood donations.

Dinghra also implicitly equates paying cash with the incentives that we studied; this is inaccurate. As emphasized in our article, the effects on safety and quantity may differ across different incentives and conditions. Paying donors cash, as done for plasma donations at private blood banks, is one strategy that we did not examine. What we did study is the effect of offering items (typically with values below minimum wage) to potential donors for presenting to make a whole blood donation, regardless of whether they actually donate or are found ineligible. These strategies could yield quite different results.

Finally, we agree that ethical principles should also guide discussion about blood donations. Societies define what kind of transactions, and in what form, are and are not ethically acceptable. Some of these

views may change over time, as the *Flynn v. Holder* decision allowing compensation for bone marrow suggests (6), whereas others remain unchanged (7). We believe that these debates benefit from the availability of relevant empirical evidence that includes, in the case of blood donations, the use of representative samples, actual donation behavior using standard collection procedures, and causally identified short- and long-term effects on both donations and safety.

NICOLA LACETERA,^{1,2*} MARIO MACIS,^{3,4}
ROBERT SLONIM^{4,5}

¹University of Toronto, Toronto, ON M5S 2E9, Canada. ²University of Toronto, Mississauga, Mississauga, ON L5L 1C6, Canada. ³Johns Hopkins University, Baltimore, MD 21202, USA. ⁴Institute for the Study of Labor (IZA), Bonn, 53113, Germany. ⁵University of Sydney, Sydney, NSW 2006, Australia.

*Corresponding author. E-mail: nicola.lacetera@utoronto.ca

References

1. *Human Bodies: Donation for Medicine and Research* (Nuffield Council on Bioethics, London, 2011); www.nuffieldbioethics.org/sites/default/files/Donation_full_report.pdf.
2. S. A. Glynn *et al.*, *Transfusion* 43, 7 (2003).
3. L. Goette *et al.*, *Transfusion* 49, 524 (2009).
4. C. L. van der Poel, E. Seifried, W. P. Schaasberg, *Vox Sang.* 83, 285 (2002).
5. R. Offergeld, R. Burger, *Vox Sang.* 85, 49 (2003).
6. *Flynn v. Holder*, U.S. Court of Appeals for the Ninth Circuit, No. 10-55643 (1 December 2011).
7. A. E. Roth, *J. Econ. Perspect.* 21, 37 (2007).

Returning to the Colombian Amazon

IN HIS NEWS FOCUS STORY "VENTURING back into Colombia" (2 August, p. 450), A. Regalado described new opportunities for research and unprecedented threats to biodiversity, as "no-go zones"—particularly the Colombian Amazon—become increasingly stable. As directors of an international cooperative program, Partners for Conservation in the Colombian Amazon (1), which aims to strengthen graduate education and research, we offer three suggestions for charting the return of science to the Colombian Amazon.

First, the return path should address critical gaps in biodiversity science in Colombia. Biodiversity publications accounted for roughly 30% of Colombian scientific production during the past two decades (2). However, an analysis of 5264 indexed publications on Colombian biodiversity (published between 1990 and 2011) indicated that conservation studies were rare (9%) and Amazonian departments were poorly represented (less than 10%) (2). The Amazon region and conservation-oriented research are immediate priorities for biodiversity science in Colombia.

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

Second, the return path should strengthen regional academic institutions, such as the Universidad de la Amazonía in Caquetá province. Colombia has many strong institutions in large cities, including Bogotá, Medellín, and Cali, but regional universities serve more students from Amazonian provinces. Academic programs in regional universities give limited attention to biodiversity conservation or interdisciplinary studies, often because they lack relevant capacity. Degree programs in applied biodiversity science are immediately needed at regional universities, along with support for further faculty training.

Finally, the return path should make biodiversity science part of peace building in the Colombian Amazon. The fate of natural areas—epicenters of civil conflict for decades and bastions of biodiversity—has been conspicuously absent from ongoing peace negotiations (3). Scientists must collaborate with leaders and Amazonian peoples that have cultural and livelihood ties to biodiversity. To safeguard natural resources in a possible post-conflict scenario, we must build broad awareness that



Universidad de la Amazonía.

Amazonian biodiversity is an irreplaceable resource for Colombia.

ELIZABETH P. ANDERSON^{1*} AND
JAVIER A. MALDONADO-OCAMPO²

¹School of Environment, Arts, and Society, Florida International University, Miami, FL 33181, USA. ²Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, Colombia.

*Corresponding author. E-mail: epanders@fiu.edu

References and Notes

1. This program is funded by the U.S. Agency for International Development through Higher Education for Development.
2. E. Arbelaez-Cortés, *Biodivers. Conserv.* 10.1007/s101531-013-0560-y (2013).

3. Los Parques: De las Balas a la Paz, *Semana* (2013); <http://m.semana.com/nacion/articulo/los-parques-de-las-balas-la-paz/358371-3> [in Spanish].

CORRECTIONS AND CLARIFICATIONS

Perspectives: “Blooms bite the hand that feeds them” by H. W. Paerl and T. G. Otten (25 October, p. 433). On the right side of the figure, the label above the downward arrow should be “Increased CO₂”; the label below the downward arrow should be “CO₃²⁻/HCO₃⁻”. The HTML and PDF versions online have been corrected.

Policy Forum: “Probiotics: Finding the right regulatory balance” by D. E. Hoffmann *et al.* (18 October, p. 314). In reference 11, the correct URL is www.law.umaryland.edu/ProbioticsWhitePaper. The HTML and PDF versions online have been corrected.

News: “Great presenters: Lighting up the auditorium” by J. Cohen (special section on Communication in Science, 4 October, p. 78). Although Bonnie Bassler discusses *V. fischeri* and symbiosis in presentations she gives about her work, her lab focuses on the closely related, but free living, *V. harveyi*. The HTML and PDF versions online have been corrected to reflect this.

Reports: “Constitutive μ -opioid receptor activity leads to long-term endogenous analgesia and dependence” by G. Corder *et al.* (20 September, p. 1394). On page 1396, Fig. 10 should be cited instead of Fig. 1M. On page 1397, fig. S5 should be cited instead of fig. S4. HTML and PDF versions online have been corrected.

BEHAVIOR

The Serious Business of Play

Gillian R. Brown

Some children gain pleasure from climbing trees, some spend hours with building bricks, and others enjoy pretending to be doctors or musicians. In *Play, Playfulness, Creativity and Innovation*, Patrick Bateson and Paul Martin argue that all of these play activities have in common the generation of novel combinations of actions or thoughts and that early play experiences promote creativity in later life. The authors (behavioral biologists at the University of Cambridge) draw on seemingly disparate literatures on play behavior in nonhuman animals; innovation in the human workplace; and the links among drugs, dreams, and creativity to argue that play is intricately linked to our ability to generate creative solutions to novel problems. The implications of the links between play and creativity apparently extend beyond any personal enjoyment that individuals might gain, as creative ideas that are adopted and implemented by others can have substantial impact on the direction of evolution. Play is therefore a most serious-minded activity.

Bateson and Martin begin by critically evaluating the literature on animal play behavior, for example, by unpacking the issue of how to define play. Most commonly, definitions of play rely on the absence of any immediate fitness benefit to the individual, and the benefits are assumed to emerge in later life. For instance, rough-and-tumble play in young mammals is suggested to provide “scaffolding” for aggressive behavior in adulthood, such that individuals that engaged in this type of play are, when adults, more likely to win fights and adjust to defeats than those with fewer such experiences. In some species, bouts of play are preceded by a distinctive facial expression (such as the open-mouth display of young macaque monkeys) or are accompanied by specific vocalizations (such as the tickling-induced, 50-kHz “laughter” in rats). However, the authors are sensibly cautious about an over-eagerness to label behavior as play sim-

ply because the immediate benefits are not apparent, because numerous forms of activity could then end up being included under the term. Play interactions in children, for example, are likely to involve a complex mixture of both short-term goals and later benefits, neither of which might be fully comprehended by adult observers.

Although discussions about the function of play have been relatively well rehearsed, the authors provide a novel and thought-provoking perspective on the field by arguing that, once play behavior has evolved, the benefits of play in assisting creativity could provide an additional selective advantage for playful individuals. The authors suggest that, “in the course of playing earlier in their lives, individuals dis-



cover properties of their environment that prove crucial when they are later faced with a new challenge.” For example, animals that played with objects early in life could have learned about causal relationships between physical entities and subsequently solve novel foraging tasks using this knowledge (e.g., by constructing tools). If so, researchers could predict individual differences in performance on cognitive tasks based on earlier play experiences. Accumulating strong empirical evidence for a causal link between play and later creativity in humans or other species will take time and effort.

As Bateson and Martin explain, the spread of novel behavioral patterns through a pop-

ulation depends on other individuals adopting the creative solutions. Traits that promote innovation—defined by the authors as the implementation of a novel behavior or idea in order to obtain a practical benefit—are not necessarily the same, and are not necessarily possessed by the same individuals, as traits that promote creativity. In human beings, persistence and organization help individuals implement others’ ideas and develop these

ideas into practical innovations. The authors review studies of how organizations and businesses promote creativity and innovation. This literature reveals that seeing the potential in new ideas, or devising new uses for old ideas, is just as important as the initial generation of novel ideas. While some organizations have achieved suc-

cess by providing opportunities for “playful play” (play accompanied by a positive, joyful mood) among staff, such activities must be balanced against the need to nurture ideas and bring their potential to fruition. Some academics might feel that their work environments actively stifle playfulness. However, the evidence that moderate levels of alcohol can enhance scientific creativity (*1*) will be appreciated by those who finish their working week with a visit to the local bar.

More seriously, this highly engaging book provides a novel perspective on the role of play activities that apparently lack seriousness. The clarity of prose and diversity of material covered in *Play, Playfulness, Creativity and Innovation* persuade the reader to reconsider the importance of play in childhood and beyond. For instance, Bateson and Martin point out that childhood play can introduce complexity to the behavioral repertoire and lead to selection for traits that underpin the adoption of novel behavior, thereby altering the evolutionary trajectory of our species. Playful play may be particularly important in generating creativity, and the authors entreat that adults still “have much to gain from deliberately adopting a more playful approach to life.” To use their quote from George Bernard Shaw, “We don’t stop playing because we grow old, we grow old because we stop playing.”

References

1. T. Norlander, R. Gustafson, *Creat. Res. J.* **11**, 265 (1998).

10.1126/science.1245862

The reviewer is at the School of Psychology and Neuroscience, University of St Andrews, St Mary’s Quad, South Street, St Andrews, Fife KY16 9JP, UK. E-mail: grb4@st-andrews.ac.uk

FILM: SPACE

The Joy and Fear in Space Exploration

Salman Hameed

NASA's human space program is currently adrift. The space shuttle has been retired, and American astronauts now hitch rides to the space station. Furthermore, it is unclear whether the next big program will land people on the Moon (again), a nearby asteroid, Mars—or perhaps, for the foreseeable future, nowhere at all. Desperate, space enthusiasts have to settle for high-budget science fiction coming out of Hollywood showing aliens fighting with robots or a dumbed-down and militarized *Trek* universe that abandons the idealistic spirit of exploration created by Gene Roddenberry. In this context, two new films, the low-budget *Europa Report* and the visually stunning *Gravity*, offer a much-needed breath of fresh air.

Set sometime in the middle of this century, *Europa Report* recounts the journey of a multinational team of six astronauts to Jupiter's moon Europa. The mission is sponsored by a private company that mixes the adventure of space exploration with some of the exploits of reality television. Sebastián Cordero's film falls in the "found-footage" genre. From the beginning, we know that something went wrong with the mission. Cameras installed in the spacecraft provided footage of the astronauts' journey that allows us to piece together their fate.

A mission to Europa, one of Jupiter's four large moons, makes sense. Beneath its ice-covered surface lies an ocean of water (*I*) kept liquid through subsurface volcanism fueled by the tidal forces of Jupiter. Conditions near the volcanic vents, thought to be similar to those found here on Earth, may provide a fertile environment for the origin and sustainability of life.

The film and its characters have a restrained quality. The astronauts are all depicted as competent, rational individuals making hard decisions under high pressure,

but they are also willing to sacrifice their lives to advance scientific knowledge. Watching *Europa Report* reminded me of the trials of Ernest Shackleton and other early-20th-century polar explorers. The film tries to balance the fear and the joy of the unknown, and it largely succeeds.

Despite its low budget, the film is beautifully shot. In particular, the cinematography makes clever use of eight fixed cameras in the spacecraft. The production design, for both the spacecraft and the European landscape, is outstanding. Unfortunately, with the exception of the lead engineer (played by Swedish actor Michael Nyqvist), the characters are not developed enough for viewers to care for them individually. But this is a minor quibble for a movie unequivocally celebrating the spirit of space exploration.

Europa Report largely takes place inside a spaceship. The beauty of space itself is more clearly on display in *Gravity*, which tells a relatively simple story. Two astronauts on a servicing mission to the Hubble Space Telescope (HST)—yes, this is an alternative universe in which astronauts still ride space shuttles to the telescope—are left on their own when debris from a Russian satellite damages the shuttle beyond repair and kills the crew members who had remained on board.

On its surface, this is a high-brow disaster film. However, director Alfonso Cuarón provides a spectacular immersive experience, with enough suspense to keep you at the edge of your seat throughout

the film. The breathtaking 17-minute opening sequence (shot in a single take) warrants the full price of a 3D admission. Commenting to the *New York Times* about this sequence, Cuarón explained "[w]e wanted to slowly immerse audiences into first the environment, to later immerse them into the action, and the ultimate goal of this whole experiment was for the audiences to feel as if they are a third character that is floating with our other two characters in space" (2). Indeed, for most of

us, this is as close as we can get to experiencing outer space.

Although the movement of astronauts and objects in space is very well depicted, some artistic choices made by the filmmakers led to scientific inaccuracies. For example, the orbits of HST and the International Space Station are different enough that it is nearly impossible to move from one to the other (as the astronauts in the movie end up doing). A



Toward Europa's ice.

Europa Report

Sebastián Cordero, director
Start Motion Pictures and
Misher Films, USA, 2013. 90
minutes. <http://start-media.com/start-motion-pictures/new-release/europa-report>

Gravity

Alfonso Cuarón, director
Warner Brothers, USA, 2013.
91 minutes. <http://gravity.movie.warnerbros.com>

tweet by Neil deGrasse Tyson correctly noted that outside of her suit, Sandra Bullock's hair should have been floating freely above her head in the space station's zero gravity environment. But these minor issues pale compared to the spirit and the overall experience of the film.

Where *2001*, *Solaris*, and *Contact* used the premise of outer space to explore larger philosophical questions, *Europa Report* and *Gravity* focus on the perils, dangers, promise, and gratification of space exploration. They also offer a clear reminder that outer space is largely hostile to life developed on Earth—but that is not, in itself, reason enough to stop space exploration. At one point in *Europa Report*, when asked whether walking on Europa is "creepy," an astronaut retorts, "It is cosmically astounding." We don't know when the U.S. human space program will regain its footing, but I was refreshed watching these films celebrate the joy, fear, and idealism of space exploration.

References

1. D. Stevenson, *Science* **289**, 1305 (2000).
2. www.nytimes.com/video/movies/100000002478606/anatomy-of-a-scene-gravity.html.

10.1126/science.1245655

CLIMATE CHANGE

Hell and High Water: Practice-Relevant Adaptation Science

Adaptation requires science that analyzes decisions, identifies vulnerabilities, improves foresight, and develops options.

R. H. Moss,*† G. A. Meehl, M. C. Lemos, J. B. Smith, J. R. Arnold, J. C. Arnett, D. Behar, G. P. Brasseur, S. B. Broomell, A. J. Busalacchi, S. Dessai, K. L. Ebi, J. A. Edmonds, J. Furlow, L. Goddard, H. C. Hartmann, J. W. Hurrell, J. W. Katzenberger, D. M. Liverman, P. W. Mote, S. C. Moser, A. Kumar, R. S. Pulwarty, E. A. Seyler, B. L. Turner II, W. M. Washington, T. J. Wilbanks

Informing the extensive preparations needed to manage climate risks, avoid damages, and realize emerging opportunities is a grand challenge for climate change science. U.S. President Obama underscored the need for this research when he made climate preparedness a pillar of his climate policy. Adaptation improves preparedness and is one of two broad and increasingly important strategies (along with mitigation) for climate risk management. Adaptation is required in virtually all sectors of the economy and regions of the globe, for both built and natural systems (1).

However, without the appropriate science delivered in a decision-relevant context, it will become increasingly difficult—if not impossible—to prepare adequately (2). We suggest a number of measures to hasten the development of science to correct maladaptations to current climate variability and support society's increasing need to adapt to a changing climate, drawing on lessons from experience, insights from related endeavors such as sustainability science (3), and input from scientific and stakeholder communities.

Adaptation Planning, Information Gaps, and the Need for Adaptation Science

Initial adaptation planning is occurring in some sectors, such as water resource management, forestry, insurance, and coastal zone management. A limited but growing number of states and cities are developing adaptation plans. U.S. federal agencies have implemented sustainability plans that include mitigation and adaptation (4).

There are serious science gaps, however (5, 6). In many communities, decision-makers lack climate information or the means to apply it. In others, knowledge of current or potential future impacts exists, but not in a form or context that decision-makers can assimilate or act on in advance. In still



The entrance to a garage in Lower Manhattan on 31 October, 2012, as New York City began clean-up after Hurricane Sandy.

others, engineering innovations are needed, as well as social science knowledge, to guide technology deployment and adjustments to management, investments, and public policy.

A key characteristic of emerging adaptation science is that it is both basic—in that it contributes to understanding fundamental physical, environmental, and socioeconomic research questions—and applied, because it is problem focused. Scientists and practitioners “coproduce” relevant research by jointly defining questions and maintaining frequent interactions (7). Coproduction is challenging to implement and sustain because participants often have different roles, vocabularies, interests, methods, and incentives. The effectiveness of communications and deliberative processes among scientists and practitioners requires empirical evaluation (8).

To support the wide range of necessary adjustments, we outline a comprehensive, integrated approach to research in social, physical, environmental, engineering, and other sciences. We describe adaptation science research needed to under-

stand decision processes and information requirements, identify vulnerabilities, improve foresight about climate risks and other stressors, and understand barriers and options for adaptation.

Understand Decision Processes and Knowledge Requirements

Adaptation science research must clarify what types of scientific information are required for improved decision-making. Decision-makers are concerned with cost, feasibility, social acceptance, tradition, and other factors. To close a “usability gap,” scientific information must fit into existing contexts (9). Organizational, cognitive, political, ethnographic, and decision sciences research is needed to clarify the problem, the values of participants, and the context in which the information will be applied. Understanding perception and apprehension of climate change risks is also a priority.

Identify Vulnerabilities

Research to characterize vulnerability and adaptive capacity focuses on pinpointing infrastructure, economic sectors, geo-

*Full affiliations for all authors are provided in the supplementary materials. †Corresponding author. E-mail: rhm@pnml.gov

graphic areas, population groups, and ecosystems at greatest risk of harm (10). Two challenges are to:

Improve data, methods, and scenarios for research on vulnerability and resilience of human and natural systems. A geo-referenced data system for factors such as population, economic status, preparedness, natural capital, and location of sensitive infrastructure needs to be established and maintained to identify vulnerable human communities and environments. Effective response will be aided by understanding the extent to which vulnerability arises from poverty, under-investment, environmental factors, and their interactions with climate variability and change.

Identify climate thresholds in vulnerable systems. Knowledge of climate and related thresholds, points at which fundamental transformations occur in natural or human systems as climate changes, will improve resource management and inform debates about future atmospheric greenhouse gas stabilization. Coupled with time-dependent climate scenarios, improved knowledge of climate thresholds may help in estimating when effects could occur and thus facilitate setting adaptation priorities.

Improve Foresight About Climate Hazards and Other Stressors

Physical and biological scientists must study climate processes and develop models to deliver insights about climate features, including temperature and precipitation extremes, and related processes such as evolution of ecosystems, sea level rise, and other first-order effects. Social sciences can characterize human contributions to climate change through emissions and land use and inform mechanisms for improving interactions between climate scientists and potential users (11). Research challenges include:

Understand recent and potential future changes in extreme climate events. Extremes occur on many spatial and time scales. They include heat waves, droughts, floods, storms, and other events that have major effects on human and natural systems. There is evidence that many of these extremes are intensifying (1, 12). A concerted focus on detecting changes in extremes and improving predictive products can provide important inputs to adaptation planning and preparedness.

Improve integration of weather and climate information. Common elements of initializing predictions with observations and providing probabilistic weather and climate information across time scales can consti-

Adaptation Science in Action

Scientists and practitioners are collaborating on research and applications to support climate change preparedness and address other nonclimate issues. Case examples document research and actions, such as plans and projects, but do not yet provide evidence of improved outcomes. Improved methods and data are needed for evaluating results (29).

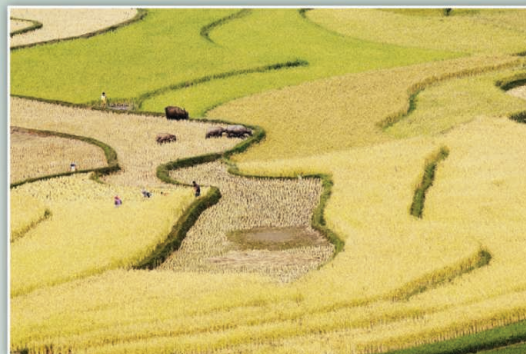
- Well before Hurricane Sandy, New York City established a task force of city officials, utilities, commercial firms, and researchers to support formulation of adaptation options for vital infrastructure.

- Major urban water utilities, university-based research centers, and private-sector firms in the United States are collaborating to pilot applications of climate science and water utility modeling applications to quantify impacts of climate change on their water systems and evaluate adaptation strategies.

- Researchers in Australia developed a plant functional trait database and modeled habitat suitability under climate scenarios for naturalized and invasive plants to enable land managers to make better-informed decisions about land management at a national and regional level.

- Researchers and local practitioners in the Vietnamese city of Hue and the Bangladeshi city of Satkhira collaborated to assess climate-related risks, identify adaptation strategies, and strengthen local capacity to manage the interaction of rapid development and climate impacts.

- Researchers worked with small-scale farmers and agricultural extension officials in sub-Saharan Africa to provide a bridge between scientific and indigenous knowledge of drought onset and coping strategies to improve delivery of drought prediction information for vulnerable, rain-fed farming operations.



tute a unified approach for weather forecasts and climate predictions (13, 14). Decadal climate predictions with next-generation, high-resolution global climate models [e.g., (15)] have the potential to produce probabilistic near-term climate information over the next decade and improve insight into future conditions to which human societies will have to adapt (16). Research is needed to formulate methods for presenting global climate model information in probabilistic form and applying that information to risk assessment and management (17).

Tailor climate information to facilitate its application in decision-making. Sustained interactions among researchers and decision-makers are needed not only to understand how climate affects assets or resources but also to identify how climate information can be used in decisions (18). In addition to global climate models, other tools can produce relevant information, such as less computationally intensive intermediate-complexity models [e.g., to assess uncertainty (19)], qualitative scenario planning approaches (20), and decision-analytic approaches to use climate model information in ways that supplement conventional scenario-led studies [e.g., (17)]. Climate and decision scientists can tailor information for application through downscaling of climate model data [e.g., (21)] or running fully dynamical nested numerical models

at regional or finer grid spacings [e.g., the North American Regional Climate Change Assessment Program (22)].

Establish climate information services at the national and international level to translate and communicate adaptation science to public and private sector decision-makers. The Global Framework for Climate Services (23) provides a foundation for climate services being developed in many countries. Development of climate services should engage decision-makers, researchers, and others to ensure that products are relevant, uncertainties are explicit, and a portfolio of products that combine monitoring and projections are useable to decision-makers. The U.S. National Academy of Sciences has emphasized the need for climate services (24), and the U.S. Global Change Research Program (USGCRP) has made climate adaptation science that would use climate information a focus of its 10-year plan (25). Current funding levels are inadequate and declining, and incentives and means to engage with stakeholders are lacking, inhibiting service development and delivery (5, 26).

Identify Barriers, Broaden the Range of Adaptation Options, and Promote Learning

Options need to be studied to guide adjustments in technology, management practices, public policy, standards, institutions, gover-

nance, and incentives to facilitate adaptation. Four priorities are to:

Identify, explain, and explore opportunities to overcome barriers to adaptation. Existing laws, regulations, and other practices can limit adaptation. These include legal and financial mechanisms, from local to international jurisdictions. Research is needed to identify these barriers and develop solutions to minimize or surmount them.

Develop technologies and performance standards. A changed climate system with conditions never before experienced by human societies may alter effectiveness of historically successful adaptation approaches and limit types of adaptation (27). Adaptation options are needed that are robust to different climate and socioeconomic development pathways, and are thus effective in both the short-term and the long-term (e.g., improved water distribution or desalination systems, and advances in electricity generation, transmission, and distribution). Organizations and processes for establishing manufacturing, building, and other standards (e.g., the International Organization for Standardization) could contribute to the development of adaptation options.

Develop indicators and monitoring and evaluation systems. Monitoring and evaluation will help track progress in enhancing resilience, prioritizing adaptation funding, and evaluating effectiveness of adaptation options. For adaptation options related to infrastructure and some natural resources, relevant indicators are relatively easy to quantify. For measures focused on governance or livelihoods, indirect proxy variables must be defined. Case studies dominate adaptation evaluations, with little comparative research on implementation (28).

Learn from experience. A number of knowledge platforms are beginning to catalog and evaluate experiences to promote information exchange and learning. Learning and disseminating of lessons needs to be accelerated to improve decision-making. Enabling private companies to share experiences without damaging commercial interests is a particular challenge.

Measures to Establish Adaptation Science

First steps in establishing institutions to conduct and support adaptation science are taking place. In the United States, “mission” agencies—including the Environmental Protection Agency, the National Oceanic and Atmospheric Administration (NOAA), and the Department of the Interior—have supported the use of scientific information in adaptation. The Regional Integrated Sciences

and Assessments Program, Climate Science Centers, and other efforts aim at developing a collaborative framework between research and practice (29). The European Union has provided research funding and launched an information portal (www.eea.europa.eu/themes/climate/european-climate-adaptation-platform-climate-adapt). Germany, the United Kingdom, and other countries are moving forward to establish services (e.g., www.climate-service-center.de). Australia has made adaptation a priority (www.csiro.au/Outcomes/Climate/Adapting.aspx).

Advances in adaptation science will not happen without two transformational changes: (i) an institutional structure to catalyze, coordinate, evaluate, and encourage the use of advances in adaptation science; and (ii) an increase in funding. National Research Council reports [e.g., (6)] and recommendations of a U.S. federal interagency committee (30) provide detailed recommendations that would help advance climate adaptation science.

One U.S. approach could be a national institute on climate preparedness composed of research and applications centers for adaptation in priority sectors or challenges. The institute would be geographically distributed and function in some ways similar to the National Institutes of Health. It would build on the current USGCRP, agency sectoral or regional research centers, and regional nodes for the National Climate Assessment’s sustained assessment process. Providing sufficient resources for collaboration and learning would facilitate research on coupled systems and offer an opportunity to better connect adaptation science to interagency programs.

More broadly, sustained support for problem-oriented fundamental research on adaptation needs to be increased at research agencies. A particular challenge is to develop effective approaches to learn from adaptation practice as well as published research. Progress toward achieving the goal of adapting to changing climate will require demonstrating tangible benefits for society by connecting research and applications.

References and Notes

- IPCC, *Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation*, C. B. Field et al., Eds. (Cambridge Univ. Press, Cambridge, UK, and New York, NY, USA, 2012).
- R. A. Kerr, *Science* **334**, 1052 (2011).
- W. C. Clark, N. M. Dickson, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 8059 (2003).
- R. Bierbaum et al., *Mitig. Adapt. Strategies Glob. Change* **18**, 361 (2013).
- National Research Council, *America’s Climate Choices: Adapting to the Impacts of Climate Change* (National Academies Press, Washington, DC, 2010).
- National Research Council, *America’s Climate Choices: Advancing the Science of Climate Change* (National Academies Press, Washington, DC, 2010).
- L. Dilling, M. C. Lemos, *Glob. Environ. Change* **21**, 680 (2011).
- N. Pidgeon, B. Fischhoff, *Nat. Clim. Change* **1**, 35 (2011).
- M. C. Lemos, C. J. Kirchhoff, V. Ramprasad, *Nat. Clim. Change* **2**, 789 (2012).
- P. C. Stern et al., *Nat. Clim. Change* **3**, 607 (2013).
- National Research Council, *America’s Climate Choices: Informing an Effective Response to Climate Change* (National Academies Press, Washington, DC, 2010).
- F. W. Zwiers et al., in *Climate Science for Serving Society*, G. R. Asrar, J. W. Hurrell, Eds. (Springer, Dordrecht, NL, 2013), pp. 339–389.
- T. N. Palmer, F. J. Doblas-Reyes, A. Weisheimer, M. J. Rodwell, *Bull. Am. Meteorol. Soc.* **89**, 459 (2008).
- J. Hurrell et al., *Bull. Am. Meteorol. Soc.* **90**, 1819 (2009).
- D. M. Smith et al., *Clim. Dyn.* (2012). 10.1007/s00382-012-1600-0
- G. A. Meehl et al., *Bull. Am. Meteorol. Soc.* (2013). 10.1175/BAMS-D-12-00241.1
- C. Brown, W. Werick, W. Leger, D. Fay, *J. Am. Water Resour. Assoc.* **47**, 524 (2011).
- S. Tang, S. Dessai, *Weather Clim. Soc.* **4**, 300 (2012).
- F. Kucharski et al., *Bull. Am. Meteorol. Soc.* **94**, 25 (2013).
- E. L. Tompkins, R. Few, K. Brown, *J. Environ. Manage.* **88**, 1580 (2008).
- R. L. Wilby, H. J. Fowler, in *Modelling the Impact of Climate Change on Water Resources*, 1st ed., F. Fung, A. Lopez, M. New, Eds. (Blackwell Publishing Ltd., Chichester, West Sussex, 2011), pp. 34–85.
- L. O. Mearns et al., *Bull. Am. Meteorol. Soc.* **93**, 1337 (2012).
- C. Hewitt et al., *Nat. Clim. Change* **2**, 831 (2012).
- National Research Council, *A National Strategy for Advancing Climate Modeling* (National Academies Press, Washington, DC, 2012).
- USGCRP, National Global Change Research Plan 2012–2021: A Strategic Plan for the U. S. Global Change Research Program. (2012). Available at: <http://library.globalchange.gov/u-s-global-change-research-program-strategic-plan-2012-2021>
- National Research Council, *Informing Decisions in a Changing Climate* (National Academies Press, Washington, DC, 2009).
- K. Dow et al., *Nat. Clim. Change* **3**, 305 (2013).
- S. C. Moser, M. Boykoff, Eds., *Successful Adaptation to Climate Change: Linking Science and Practice in a Rapidly Changing World*. (Routledge, London, 2013).
- R. S. Pulwarty et al., in *Integrated Regional Assessment of Global Climate Change*, C. G. Knight, J. Jager, Eds. (Cambridge Univ. Press, Cambridge, UK, 2009), pp. 367–393.
- Council on Environmental Quality (CEQ), Progress Report of the Interagency Adaptation Task Force: Recommended Action in Support of a National Climate Change Adaptation Strategy (White House Council on Environmental Quality, Washington, DC, 2010).

Acknowledgments: The authors acknowledge the Aspen Global Change Institute (AGCI) and its director, John Katzenberger, for hosting a workshop that laid the foundations for this paper, along with the attendees who contributed to discussions. A portion of this research was supported by the Office of Science, Biological and Environmental Research, U.S. Department of Energy. Funding for the AGCI workshop was provided by NASA. The views expressed in this article are those of the authors and do not necessarily reflect the positions of their institutions.

Supplementary Materials

www.sciencemag.org/content/342/6159/696/suppl/DC1

10.1126/science.1239569

CANCER

Fusion for Moving

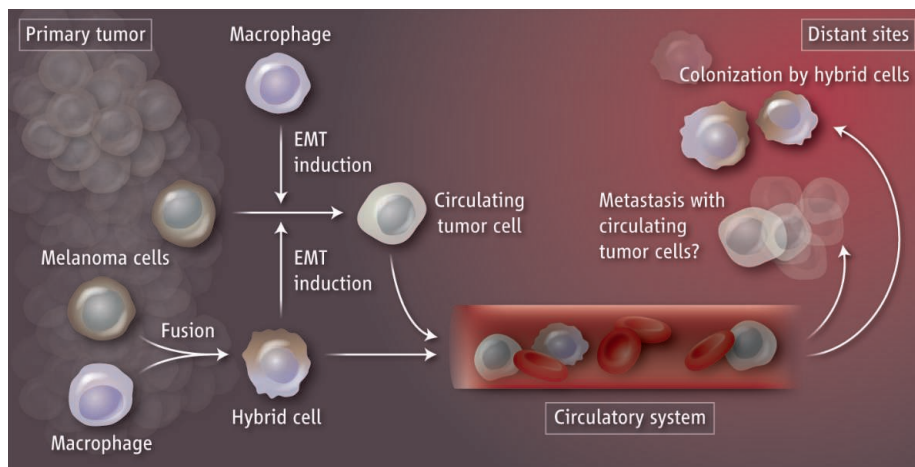
Gary A. Clawson

Most lethal cancers are carcinomas, which arise from epithelia, tissues composed of cells that touch each other, usually forming continuous sheets that line the cavities and surfaces of structures throughout the body. The clinical problem with carcinomas is that they can spread to other parts of the body. Exactly how they develop this capacity is not completely clear, although a number of routes to metastasis have been proposed. Most prominent is the idea that cancer cells undergo a transition from an epithelial state to a more primitive mesenchymal state. In this transition, cell-cell adhesion is lost and cells gain the ability to migrate. Among the other proposed metastatic mechanisms is the idea that a tumor cell could fuse with a mobile cell type and then travel to another site in the body to establish cancer. Although this notion was raised about 100 years ago, it never received widespread attention. This theory may have gained some new traction through a recent study of melanoma metastasis (1).

In 1911, German pathologist Otto Aichel proposed that cancer cells fuse with white blood cells to create mobile hybrids that co-opt the former's ability to travel through the circulatory and lymphatic systems, while retaining cancer's capacity for unbridled growth. The idea of such an astonishing hybrid has had support from *in vitro* cell fusion experiments and from animal studies in which the observed metastasis stemmed from the implantation of such hybrid cells. These hybrids traditionally have been thought to undergo "nuclear reprogramming" to maintain their cancer-initiating properties (2). The recent discovery in cancer patients of circulating tumor cells expressing both carcinoma and leukocyte cell markers also points to fusion events between bone marrow-derived cells and tumor cells. But myeloid traits are often found in many cancers, and it has remained unclear how frequently this mechanism might give rise to tumors. Because tumor cells and bone marrow-derived cells in an individual cannot be easily distinguished genetically, it has been difficult to confirm the fusion mechanism in human cancer.

Gittlen Cancer Research Foundation, Department of Pathology, and Department of Biochemistry & Molecular Biology, Hershey Medical Center, Pennsylvania State University, Hershey, PA 17033, USA. E-mail: gac4@psu.edu

Tumor cell fusion with white blood cells has been a long-standing theory of metastasis—is there sufficient evidence yet?



Hybrids on the move. Tumor-associated macrophages may fuse with epithelial cancer cells (or nonepithelial cells such as melanoma) at the site of the primary tumor. These hybrids might then induce the EMT transition in some cancer cells, allowing them to escape into the circulatory system (along with the hybrid cells) and colonize distant organ sites.

Lazova *et al.* (1) recently proposed that an apparent metastatic melanoma lesion in a patient (a brain metastasis) arose from the fusion of a bone marrow-derived cell with a tumor cell, which occurred after a bone marrow transplant (the cancer patient received a transplant from his brother for treatment of B cell lymphoma). The authors examined genomic DNA from the brain melanoma and found the presence of donor and patient genes in cancer cells throughout the brain tumor, arguing for bone marrow-derived cell-tumor cell fusion as the origin of tumor-initiating cells. However, the study did not definitely establish that such a fusion caused the metastatic lesion. The authors were not able to identify a "primary" melanoma tumor at any other site. Unknown primaries are sometimes a necessary clinical default, but melanocytes (unlike pancreatic or colorectal epithelial cells) are also found in various locations in the brain, so the brain lesions identified by the authors could be an example of how bone marrow-derived cell-tumor cell fusions could produce a primary tumor but have nothing to do with metastasis *per se*.

The more popular alternative to explain metastasis is the acquisition of epithelial-mesenchymal transition (EMT) traits that confer migratory and invasive properties to cancer cells, allowing them to escape from the primary tumor microenvironment into the circulatory systems (circulating tumor cells)

(3, 4). But various aspects of this phenomenon are also not clear, such as the identity and nature of the cancer-initiating cells or their phenotypic characteristics. It is possible that cancer stem cells—those cells in a tumor that can self-renew and give rise to all cell types found in a particular tumor (5)—“accompany” tumor cells (which have arisen through the EMT process) that can escape from the primary tumor. If this is the case, the principle of Occam’s razor (which favors the simplest explanation for a phenomenon) would ask where the need is to postulate another population of cancer-initiating cells—the bone marrow cell-tumor cell hybrids.

The cell fusion and cancer stem cell theories represent possibilities that are generally considered distinct and seem to be considered as “linear” events, each sufficient to ultimately produce metastatic lesions. However, they may well be intertwined (see the figure). At the site of the primary tumor, fusions of epithelial cancer cells and tumor-associated macrophages could actually occur quite frequently. Because macrophages are among the most mobile cells in the body, these hybrids would have “professional-grade” migratory or invasive capability and could widely disseminate throughout the circulatory system and colonize distant sites as an initial step in the metastatic cascade.

The first implication of this scenario is that it should be possible to find macro-

phage–tumor cell fusions in the circulation, or at multiple distant organ sites, before the establishment of epithelial cancer cells per se. Although there is experimental support for the presence of hybrids in tissues without metastases, the extent of this phenomenon has not been systematically explored. For example, macrophage–tumor cell fusions have been characterized in sentinel lymph nodes in melanoma patients who otherwise lacked histologically detectable melanoma (6). Circulating hybrids also have been reported in colorectal cancer patients and pancreatic cancer patients (7); the latter is consistent with the “motility” gene signature for circulating pancreatic tumor cells (8). These circulating hybrids were identified in cancer patient blood samples in which no circulating tumor cells were detected by standard tests. Moreover, the hybrids expressed macrophage migration inhibitory factor, whose corresponding mRNA is a biomarker for an early stage in melanoma. This inflammatory cytokine has pleiotropic effects in a large number of carcinomas (9, 10), particularly in pancreatic and colorectal cancer (11, 12). Further, mRNA encoding migration inhibitory factor is preferentially released (along with other factors) from tumor cells in exosomes to modulate “premetastatic” organ sites for subsequent colonization (13). A reasonable case can therefore be made for the idea that macrophage–tumor cell fusions formed at the primary tumor site could travel to distant sites in the body and create local microenvironments conducive to the development of metastases through the release of procarcinogenic factors.

On the other hand, at the site of the primary tumor, macrophage migration inhibitory factor (originating from cancer cells, hybrids, and tumor-associated macrophages) can induce the EMT and enhance tumor aggressiveness in many cancer types, particularly pancreatic cancer (14). In turn, tumor-associated macrophages provide support for cancer stem cells because targeting these macrophages for destruction in primary tumors has been shown to decrease cancer stem cells (15).

Many questions remain about how fusion occurs, its frequency in cancer, and its role in spreading cancer metastatically. Macrophage–tumor cell fusions may well function in the metastatic cascade not only at the site of the primary tumor but also at distant organ sites, where they could help prepare the microenvironment for efficient colonization. It may be that if macrophage–tumor cell fusions or bone marrow–derived

cell–tumor cell fusions occur frequently, they would not have to assume the role of tumor-initiating cells, perhaps leaving that to a companion cancer stem cell subpopulation. In addition, the presence of such hybrids in the circulation could provide an early diagnostic window of opportunity, before metastatic lesions develop.

References and Notes

1. R. Lazova *et al.*, *PLOS ONE* **8**, e66731 (2013).
2. J. M. Pawelek, A. K. Chakraborty, *Nat. Rev. Cancer* **8**, 377 (2008).
3. M. Yu *et al.*, *Science* **339**, 580 (2013).
4. G. Kallergi *et al.*, *Breast Cancer Res.* **13**, R59 (2011).
5. S. Kraljevic Pavelic, M. Sedic, H. Bosnjak, S. Spaventi, K. Pavelic, *Mol. Cancer* **10**, 22 (2011).
6. E. Itakura, R. R. Huang, D. R. Wen, A. J. Cochran, *Am. J. Surg. Pathol.* **35**, 1657 (2011).

7. G. A. Clawson *et al.*, *PLOS ONE* **7**, e41052 (2012).
8. G. Sergeant, R. van Eijdsen, T. Roskams, V. Van Duppen, B. Topal, *BMC Cancer* **12**, 527 (2012).
9. H. Conroy, L. Mawhinney, S. C. Donnelly, *Q. J. Med.* **103**, 831 (2010).
10. T. Yuan, C. Tang, M. Chen, S. Deng, P. Chen, *Genet. Mol. Res.* **12**, 10.4238/2013.January.4.3 (2013).
11. A. Denz *et al.*, *J. Surg. Res.* **160**, 29 (2010).
12. N. Bitarte *et al.*, *Stem Cells* **29**, 1661 (2011).
13. S. Rana, K. Malinowska, M. Zöller, *Neoplasia* **15**, 281 (2013).
14. N. Funamizu *et al.*, *Int. J. Cancer* **132**, 785 (2013).
15. J. B. Mitchem *et al.*, *Cancer Res.* **73**, 1128 (2013).

Acknowledgments: Supported by National Cancer Institute grant CA170121 and by Tobacco CURE funds from the Pennsylvania Department of Health (the Department disclaims responsibility for any analyses, interpretations, or conclusions).

10.1126/science.1244270

DEVELOPMENT

A Stem Cell Perspective on Cellular Engineering

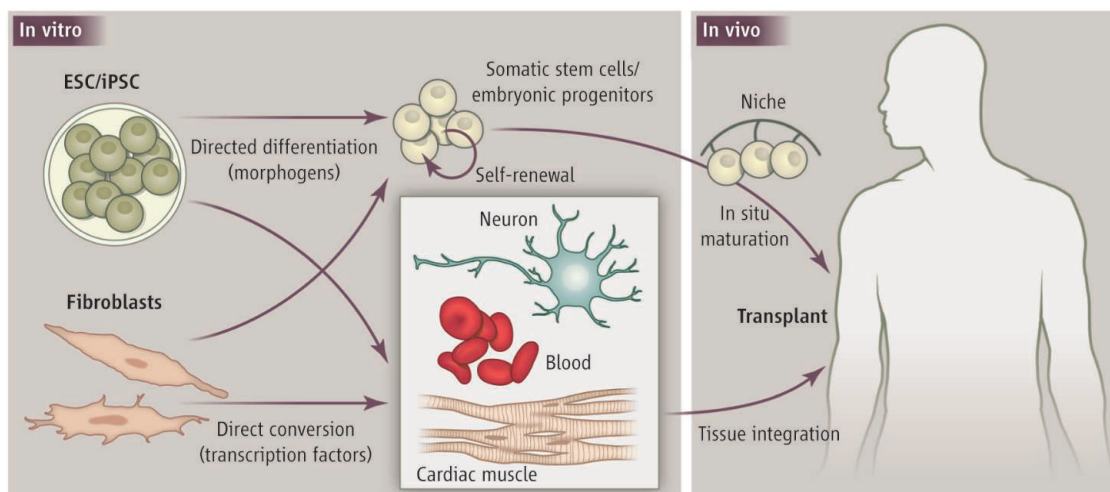
Sergei Doulatov and George Q. Daley

Alternative ways of engineering cells in vitro are being tested to realize their therapeutic potential.

A fundamental enigma in modern biology concerns the molecular rules that govern how cells establish and maintain identity during development. These rules are the key to generating therapeutic cell types in vitro and the foundation of regenerative medicine. The isolation of embryonic stem cells (ESCs) has enabled scientists to recapitulate the process of embryonic development in a dish, by directing differentiation of ESCs with combinations of morphogens and growth factors to mimic embryonic development. These experiments assume that cell and tissue fates evolve along specific paths, and once established, remain fixed. But the advent of cellular reprogramming has fundamentally altered our view of the stability of cell identity, and dramatic demonstrations of the interconversions of mature cell types have introduced the provocative idea that cell identity can be engineered to play beneficial therapeutic roles.

Stem Cell Transplantation Program, Division of Pediatric Hematology/Oncology, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Children's Hospital Boston and Dana Farber Cancer Institute; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School; Broad Institute; Harvard Stem Cell Institute; Boston, MA 02115, USA. E-mail: george.daley@childrens.harvard.edu

The discovery of induced pluripotency taught us that a somatic cell can be reprogrammed to a pluripotent state (an induced pluripotent stem cell, iPSC) by enforced expression of several transcription factors (1). Stable cell identity depends on the existence of preferred “attractor states” of the epigenome, which represent stable “valleys” within an otherwise dynamic landscape of cellular phenotypes. Cell identities often hinge on bistable switches, which are governed by sets of transcription factors that promote and reinforce one lineage program while antagonizing alternatives, as in the segregation of the inner cell mass and trophoblast in the early embryo. Development proceeds until cell identity is stabilized in a terminally differentiated state. A challenge to the notion of fixed cell identities is the recent finding that the expression of heterologous lineage factors can enable direct conversions between cell types (2). Generally, direct conversion occurs without involving normal developmental intermediates. Although direct conversion aims to reproduce physiologic mechanisms of lineage specification, it can lead to aberrant cell types without a clear equivalent in nature. Novel computational methods will be needed to assess the fidelity of reported conversions and to diagnose



Engineering cell identity. Directed differentiation of ESCs and iPSCs relies on morphogens and growth factors to mimic embryonic development. Direct conversion uses transcription factors to force somatic cells to transit between cell states, generally without involving normal developmental intermediates. Differentiation or conversion can follow one of two paradigms. Functionally mature cell types can be produced in a dish, but they may have a limited life span after transplantation in vivo. Alternatively, somatic stem cells for the target tissue are derived and transplanted; they are then stably maintained in their native niches, self-renew indefinitely, and differentiate to tissue-specific cell types.

which gene sets remain incompletely converted or aberrantly activated. Should these engineered cells function effectively, they may prove highly valuable for medical applications, but their safety must first be proven.

Directed differentiation from pluripotent stem cells (PSCs) and direct conversion from other somatic cells have emerged as two powerful paradigms for manipulating cell fate (see the figure) (2, 3). What has received less attention is the place that somatic stem cells occupy in these paradigms. Most mature cell types in the body are not self-renewing; they are either continuously replenished from somatic stem cells (e.g., hematopoietic, intestinal, and epidermal) or are replaced slowly or intermittently in response to injury (e.g., muscle satellite cells). There are important differences that concern the rate of turnover (high turnover in blood, skin, and intestine, versus low turnover in the heart and brain), and exceptions (some cell types, such as pancreatic beta cells and memory T cells, renew without stem cells). For many tissues, an important question is whether, for therapeutic purposes, the goal of directed differentiation or direct conversion should be to generate functionally mature cells or to generate immature somatic stem cells.

Directed differentiation or direct conversion are typically aimed at generating a mature cell type, such as a contracting cardiomyocyte or a dopaminergic neuron. Although this approach is most commonly pursued today, its fundamental limitation is that mature cells may be postmitotic or lack significant proliferative potential, which limits

both the number of cells that can be obtained in a dish and their ability to expand or even simply persist in situ after transplantation. Cardiomyocytes and neurons are long-lived cell types, which favors their maintenance in vivo, yet efforts to deliver these cells into the heart muscle or brain are hampered by the inability of mature cells to properly integrate and persist in the tissue.

Another approach is to generate somatic stem cells that can be expanded and matured in a dish or transplanted into the host tissue. Target stem cells include hematopoietic (HSCs), intestinal (ISCs), mesenchymal (MSCs), neural, cardiac progenitor, skeletal muscle progenitors and satellite cells, and bronchio-alveolar lung stem cells. Instead of promoting maturation, protocols strive to capture and propagate the stem cell state. The success of transplantation is facilitated by the natural capacity of stem cells for long-term persistence and renewal in their native niches, ensuring a lasting benefit of the graft. There is also increasing evidence for many cell types that in vivo maturation produces cells with improved function relative to those matured in a dish (4). This strategy is promising, yet it faces a number of difficulties. Stem cells are rare, and mechanisms of self-renewal are poorly understood, complicating efforts to expand and maintain stem cells.

These challenges are especially evident in attempts to regenerate the hematopoietic lineage from PSCs. Because of their ability to fully reconstitute the blood system upon transplantation, HSCs are an extremely valuable therapeutic cell type. Numerous

highlights the need for novel approaches to generating somatic stem cells.

One alternative may be conversion from related lineages that minimize the “epigenetic distance” to a desired cell type, providing the context for more precise cell fate alterations. Myeloid precursors differentiated from human PSCs can be respecified into transplantable multipotential progenitors with the combination of the transcription factors ERG, HOXA9, and RORA (8). A similar approach may be applied to other tissues by introducing stem cell transcription factors into progenitors or transient amplifying cells from PSCs or primary sources. In vitro culture conditions are impoverished relative to the three-dimensional, dynamic milieu of the developing embryo. This environment may have to be recapitulated in vitro to capture and maintain stem cells. For instance, ISCs require Wnt signals from neighboring Paneth cells; ISCs aggregated with Paneth cells efficiently form three-dimensional organoids (9). Hepatic endoderm differentiated from human PSCs can aggregate with primary MSCs and human umbilical vein endothelial cells, recapitulating organogenesis in a dish (10).

In cases where the niche is not known or is difficult to recapitulate in vitro, conversion may be carried out directly in vivo. Delivery of the set of transcription factors Gata4, Mef2c, and Tbx5 into the mouse heart induces the conversion of cardiac fibroblasts into cardiomyocytes that show improved function relative to in vitro reprogrammed counterparts (4). However, trans-

differentiation protocols have been established that attempt to mimic the conditions of hematopoietic ontogeny (1, 5, 6). Direct conversion from fibroblasts using a combination of four transcription factors—cFos, Gata2, Gfi1b, and Etv6—has also recently been reported (7). These protocols generate large numbers of hematopoietic progenitors, but not bona fide HSCs. Even if HSCs could be generated, no conditions exist to faithfully preserve stem cell potential during prolonged culture. The long-standing struggle to generate transplantable HSCs

lation to human may not always be direct. For instance, *Hoxb4* converts blood precursors from mouse ESCs into HSC-like cells (11), yet extensive efforts to adapt *HOXB4* for human cells have been largely unsuccessful (12). The advent of powerful genome-editing technologies enables the creation of transgenic human lines harboring defined factors or stem cell reporters. Combined with improved xenotransplantation models, engineering directly in human cells with functional validation in engrafted mice is an attractive approach.

Moving forward, the stem cell research community must creatively apply directed differentiation and direct conversion toward engineering clinically valuable cells, targeting the generation of either mature functional cells or stem cells, depending on the anticipated

clinical application and guided by the cell type and tissue of interest. For long-lived cells such as cardiomyocytes and neurons, integration of mature cells into the tissue remains a viable option. Still, the stem cell approach should be explored, because functional tissue integration may be more permissive for neural or cardiac progenitors. For short-lived tissues such as blood, mesenchyme, skin, or intestinal epithelium, the generation of somatic stem cells will be a prerequisite for stable engraftment and prolonged tissue reconstitution. Such advances will require novel markers and reporter lines, deeper understanding of stem cell-specific transcription factors, and screening strategies formulated to derive and detect rare stem cells. The fundamental demonstration of the past decade that cell identity can be molded to our specifications has created

an unprecedented opportunity to create rare patient-specific cell types and even tissues. The decade to come will establish whether this revolution in basic science will have a lasting impact on medicine.

References

1. K. Takahashi *et al.*, *Cell* **131**, 861 (2007).
2. T. Vierbuchen, M. Wernig, *Mol. Cell* **47**, 827 (2012).
3. S. A. Morris, G. Q. Daley, *Cell Res.* **23**, 33 (2013).
4. L. Qian *et al.*, *Nature* **485**, 593 (2012).
5. M. Kennedy *et al.*, *Cell Rep.* **2**, 1722 (2012).
6. K. D. Choi *et al.*, *Cell Rep.* **2**, 553 (2012).
7. C.-F. Pereira *et al.*, *Cell Stem Cell* **13**, 205 (2013).
8. S. Doulatov *et al.*, *Cell Stem Cell* **13**, 459 (2013).
9. T. Sato *et al.*, *Nature* **469**, 415 (2011).
10. T. Takebe *et al.*, *Nature* **499**, 481 (2013).
11. M. Kyba, R. C. Perlingeiro, G. Q. Daley, *Cell* **109**, 29 (2002).
12. L. Wang *et al.*, *J. Exp. Med.* **201**, 1603 (2005).

10.1126/science.1238363

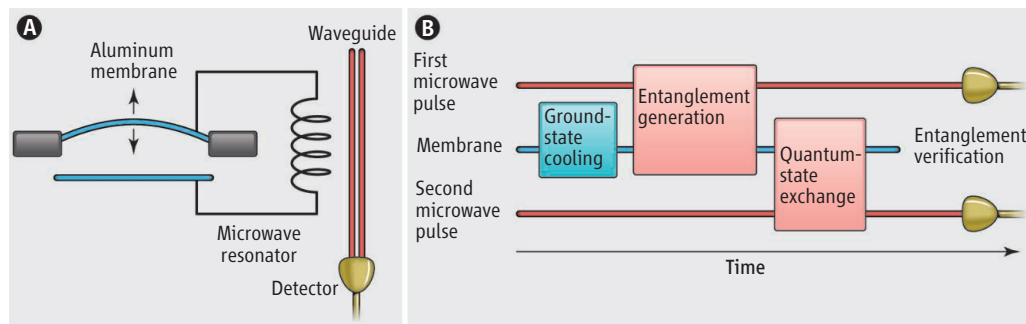
PHYSICS

Quantum Mechanics Tackles Mechanics

Klemens Hammerer

Quantum theory describes the physical cosmos at atomic and smaller scales, but can we apply quantum mechanics to large, distributed mechanical structures? Several recent experiments have shown that we can observe quantum dynamics of nano- and micro-mechanical oscillators. On page 710 of this issue, Palomaki *et al.* (1) report the controlled generation and verification of quantum entanglement of a mesoscopic mechanical device (a mechanical oscillator) with an electromagnetic microwave field. Entanglement is considered to be the distinguishing feature that separates quantum from classical physics. Only the properties of the entire system have precise values, and the mechanical resonator and the microwave field must be described by one compound quantum-mechanical wave function. No such wave functions can be assigned to either of the subsystems separately.

Institute for Theoretical Physics and Albert Einstein Institute, Leibniz University, Hannover, Germany. E-mail: klemens.hammerer@itp.uni-hannover.de



Massive entanglement. (A) The experimental setup of Palomaki *et al.* used a vibrating micrometer-sized aluminum membrane integrated in a microwave resonator. The resonator was driven through an adjacent waveguide. The amplitudes of fields emanating from the microwave cavity were detected. (B) The protocol for entanglement generation and verification is illustrated: The membrane mechanical oscillator was initialized in its quantum-mechanical ground state. An initial pulse became entangled with the oscillator, and the quantum state of the oscillator was swapped to a second pulse. The train of two pulses was detected, and the entanglement was verified from the cross correlation of the subsequent measurements.

In the experiment performed by Palomaki *et al.*, a thin circular aluminum membrane (100-nm thick and 15 mm in diameter) was suspended in a fixed frame and was free to oscillate like a drumhead. The fundamental mode of this mechanical oscillator is the one that became entangled with the microwave field. The aluminum also served as one end of a parallel plate capacitor that was integrated into a resonant circuit with a characteristic frequency in the microwave domain at a frequency of $2\pi \times 8$ GHz (see the figure,

panel A). The mechanical motion of the drum mode changed the capacitance and with it the resonance frequency of the microwave cavity. This mechanism resulted in an extremely strong mutual coupling between the mechanical and the microwave resonator.

The coupling happened on a time scale faster than the characteristic time scale on which quantum states of the two resonators could be destroyed by uncontrolled interactions with their respective environments. At the experimental temperature of 20 mK, the

coherence of quantum states could be preserved up to times on the order of hundreds of microseconds, well beyond the time scale of the strong mutual coupling of the mechanical oscillator and the microwave resonator.

In addition to the large strength of coupling, the authors also exploited its rich controllability: By driving the resonator circuit with microwave fields of suitable frequency, they could select one out of a number of different effects induced by the coupling: When the driving field had a frequency below the resonance of the microwave cavity, the net effect was to cool the mechanical resonator. Even the quantum mechanical ground state could be reached with this method. The cooling process can be regarded also as a swap between the thermal state of the mechanical oscillator and the quantum state of the incoming microwave field in vacuum. Both processes, cooling to the mechanical ground state and coherent state exchange between the mechanical oscillator and the microwave field, have been demonstrated separately in the same laboratory with a similar system (2, 3).

When the driving field was tuned above the cavity frequency, the opposite effect prevailed, and both systems were heated up. However, the blurring of the properties of the subsystems and their seeming thermalization are just facets of the entanglement generated between the motion of the mechanical oscillator and the microwave field. Far from being thermally excited, the state of the compound system had almost zero entropy. It was actually very close to the famous entangled state introduced almost 80 years ago by Einstein,

Podolsky, and Rosen in their discussion of the ostensible incompleteness of quantum mechanics (4).

Palomaki *et al.* combined all of these tools for quantum state engineering in a protocol (5) that proceeded in three steps (see the figure, panel B). They first cooled the mechanical oscillator to its ground state. The driving field was then swept above the resonance frequency to create an entangled state between motion and the microwave field. The entangled field left the cavity in the form of a short pulse and was guided to a detector where the field amplitudes were measured. Finally, after a short delay, the state of the mechanical oscillator was swapped to another pulse of the microwave field that was again guided to the detector. From the subsequent measurements of the two pulses emanating from the microwave cavity, the authors obtained complete information about the quantum state. They could precisely track how the properties of each of the subsystems became increasingly uncertain while the overall state stayed pure and exhibited strong correlations. Thus, the distinguishing quantum property of entanglement was observed in a system with a large mechanical degree of freedom.

The accomplishment by Palomaki *et al.* should be seen in the context of a series of other experiments that have tested and demonstrated implications of quantum theory in systems of micrometer-sized mechanical oscillators coupled to microwave or optical fields. Beyond ground state cooling (2, 6) and coherent state exchange (3, 7), optomechanical systems have also been used to observe

slow light (8, 9) and to verify some decade-old predictions of quantum measurement theory (10), namely, the generation of ponderomotive squeezing of light (11, 12) and the occurrence of measurement back action noise in linear position sensing (13, 14). These experiments, together with the newly observed optomechanical entanglement, finally achieve what has been formulated as a vision by Schwab and Roukes (15); they have put mechanics into quantum mechanics. It appears as if quantum theory, bizarre as it is, applies at any physical scale. It might just be getting dramatically more difficult to unveil it as we enter the truly macroscopic regime, but who knows how far we can go?

References

1. T. A. Palomaki, J. D. Teufel, R. W. Simmonds, K. W. Lehnert, *Science* **342**, 710 (2013); 10.1126/science.1244563.
2. J. D. Teufel *et al.*, *Nature* **475**, 359 (2011).
3. T. A. Palomaki, J. W. Harlow, J. D. Teufel, R. W. Simmonds, K. W. Lehnert, *Nature* **495**, 210 (2013).
4. A. Einstein, B. Podolsky, N. Rosen, *Phys. Rev.* **47**, 777 (1935).
5. S. G. Hofer, W. Wieczorek, M. Aspelmeyer, K. Hammerer, *Phys. Rev. A* **84**, 052327 (2011).
6. J. Chan *et al.*, *Nature* **478**, 89 (2011).
7. A. D. O'Connell *et al.*, *Nature* **464**, 697 (2010).
8. S. Weis *et al.*, *Science* **330**, 1520 (2010).
9. A. H. Safavi-Naeini *et al.*, *Nature* **472**, 69 (2011).
10. V. B. Braginsky, F. Y. Khalili, *Quantum Measurement* (Cambridge Univ. Press, Cambridge, 1995).
11. D. W. C. Brooks *et al.*, *Nature* **488**, 476 (2012).
12. A. H. Safavi-Naeini *et al.*, *Nature* **500**, 185 (2013).
13. T. P. Purdy, R. W. Peterson, C. A. Regal, *Science* **339**, 801 (2013).
14. K. W. Murch, K. L. Moore, S. Gupta, D. M. Stamper-Kurn, *Nat. Phys.* **4**, 561 (2008).
15. K. C. Schwab, M. L. Roukes, *Phys. Today* **58**, 36 (2005).

10.1126/science.1245797

PHYSICS

Cold-Atom Thermoelectrics

Tero T. Heikkilä

Thermoelectric devices can convert temperature differences into electric power, or cool materials by passing currents. On page 713 of this issue, Brantut *et al.* (1) demonstrate this kind of coupling between particle and heat currents in a laser-controlled cloud of lithium atoms. The precise control of such cold-atom systems allows for an accurate tuning of the thermoelectric effects that is usually not possible

in solid-state systems. Such model systems will provide a testing ground for ideas aimed at improving thermoelectric devices.

Passing a current through an electric conductor heats it up due to its inherent resistance to the current. Under suitable conditions, a small current can also create a temperature difference across a contact between two conductors, so that one part cools down and the other heats up. This Peltier effect (2) is typically weak but is used in electricity-powered portable coolers. The reciprocal effect, electrical power generation from temperature differences, the Seebeck effect (3), occurs when two dissimilar conductors are

Two coupled reservoirs of cold atoms can be used as a model system to study the thermoelectric effect.

connected in a loop and exposed to a temperature gradient. This thermopower is used especially in temperature sensors. If the efficiency of thermoelectric generation can be improved, then it could find application in power generation by harvesting the waste heat from various industrial processes. Thermoelectric effects have also found uses in various other applications, ranging from car-seat coolers and heaters to powering space missions (4).

The microscopic theory of the Peltier and Seebeck effects in metals and semiconductors has been understood for decades (5). However, the problem is that thermoelectric

Nanoscience Center, Department of Physics, Post Office Box 35 (YFL), FI-40014 University of Jyväskylä, Jyväskylä, Finland, and Low Temperature Laboratory, Aalto University, Post Office Box 15100, FI-00076 AALTO, Finland. E-mail: tero.t.heikkila@jyu.fi

devices seldom, if ever, follow this theory, mostly because of extra contributions from phonons, the vibrations of the atomic lattice constituting the materials. Such contributions are often difficult to describe without knowing the exact microscopic structure of the device. Moreover, the simplifications typically made in microscopic theories of electron systems, which work very well for the particle current, often fail for the thermoelectric effects, and hence their predictions are often rather qualitative than quantitative.

This is where the cold-atom system studied by Brantut *et al.* comes into play. Laser-

thermoelectric coefficients obtained in this experiment agree with the theoretical expectation extremely well. Such a precise agreement allows for a detailed investigation of the effects of different confinements and of disorder on the thermoelectric effects.

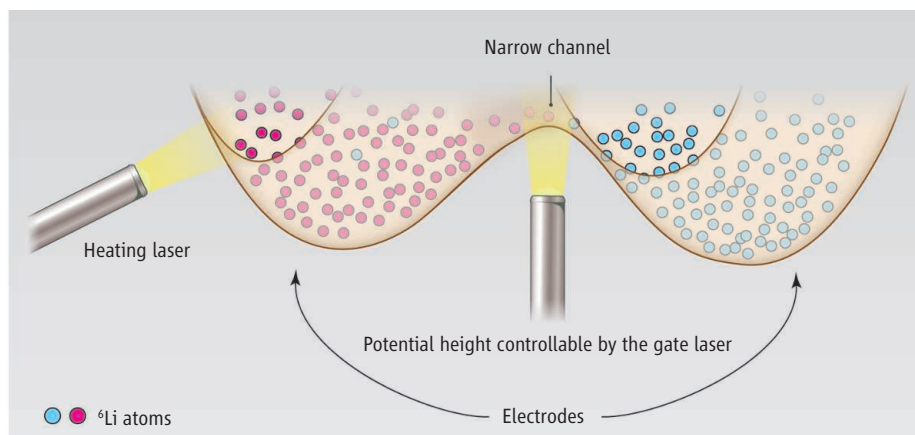
To be able to carry out these experiments, Brantut *et al.* needed to cool their gas into a very low temperature, in this case 250 nanokelvin. However, cold is a relative concept: This temperature was slightly above a quarter of the Fermi temperature, a temperature below which the gas particles are sensitive to their fermion character (that is,

contribution to the thermoelectric effect usually not present in solid-state systems.

The thermoelectric coupling in ultracold atomic gases has recently been demonstrated also in another setting (11). There, the heat was generated in tunable collisions between three atoms, which do not conserve the particle number inside the laser trap. Observing the resultant dynamics of particle and energy densities then allowed the thermoelectric coefficients to be determined. However, this method was far more indirect than that of Brantut *et al.*, and the error bars in the thermoelectric coefficients were much larger.

What is next for the thermoelectric effect in ultracold atoms? Conventional thermoelectrics are semiconductors or weak insulators featuring an energy gap (4, 12). Such energy gaps could be induced in cold-atom systems by using additional lasers to create a lattice for the atoms (6), similar to the atomic lattice present in solid-state systems. Further, the cold-atom systems could allow for a detailed investigation of the effects of interactions between the atoms on the thermoelectric effects, a notoriously difficult task in ordinary conductors. Moreover, as Brantut *et al.* suggest, the Peltier cooling of the cold atoms to an even lower temperature might allow reaching conditions where effects of strong correlations could be directly studied.

Although the thermoelectric effects have been known for almost two centuries, their efficiencies have been low. It remains to be seen if the studies in the cold-atom systems help to bring the necessary improvements so that thermoelectric power generators would help make better use of the waste heat or bring the small and reliable thermoelectric refrigerators to our kitchens.



A cool conductor. In the work by Brantut *et al.* (1), a gas with a number of ^6Li atoms is confined by two laser beams into two large regions (electrodes) separated by a narrow channel with controllable transmission. An additional laser pulse heats the atoms in one of the electrodes while the channel is closed. Opening the channel abruptly allows particle and heat flow between the electrodes. The temperature difference leads to a net particle flow from the hot to the cold electrode due to the thermoelectric effect. After a variable waiting time, the channel is closed and the temperature and particle number of the electrodes is measured. Comparison with a simple theoretical model allows the amplitude of the particle current due to the temperature difference to be determined, which then provides the thermoelectric coefficients.

confined ultracold-atom gases have emerged as a tool for simulating the various effects taking place in many-particle systems. These range from high-temperature superconductivity to strongly correlated insulating states of matter (6). One such avenue of research is the particle transport through a confined channel between two larger regions serving as electrodes. This setting mimics the charge and heat transport in electron systems (7, 8) but where the atoms in the gas replace the electrons. Earlier demonstrations showed how resistance appears at the contacts between the electrodes and a defect-free channel (9) and how the resistance of the channel drops due to the onset of superfluidity (10). Brantut *et al.* now show how the thermoelectric transport properties of the cold-atom channel can be studied by observing the particle number imbalance generated by heating one of the electrodes with an additional laser pulse (see the figure). Contrary to most solid-state realizations, the

two particles cannot occupy the same state). In metals, the corresponding temperature regime would be thousands or tens of thousands of kelvin, typically above their melting temperature. In fact, this relatively high temperature is important for the observation of a large thermoelectric effect in the cold-atom gas, because the effect is maximal for temperatures close to the Fermi temperature. This is also the reason thermoelectric effects and their efficiency in metals close to room temperature are usually quite small.

There are other principal differences in the ultracold gas experiment compared with the ordinary solid-state thermoelectric effects: Above all, there is no lattice whose vibrations would contribute either to the heat flow or to the thermoelectric power. Moreover, whereas the cold-atom systems rely on a fixed particle number inside the electrodes, this number is practically infinite in solid-state systems. The fixed number of particles in Brantut *et al.*'s experiments adds a second

References

1. J.-P. Brantut *et al.*, *Science* **342**, 713 (2013); 10.1126/science.1242308.
2. J. C. A. Peltier, *Observations sur les Multiplicateurs et sur les Piles Thermo-electriques* (1839).
3. T. J. Seebeck, *Abh. Preuss. Akad. Wiss.* **1822**, 265 (1822–1823).
4. L. E. Bell, *Science* **321**, 1457 (2008).
5. N. W. Ashcroft, N. D. Mermin, *Solid State Physics* (Brooks Cole, 1976).
6. I. Bloch, W. Zwerger, *Rev. Mod. Phys.* **80**, 885 (2008).
7. T. T. Heikkilä, *The Physics of Nanoelectronics* (Oxford Univ. Press, 2013).
8. Yu. V. Nazarov, Ya. M. Blanter, *Quantum Transport: Introduction to Nanoscience* (Cambridge University Press, 2009).
9. J.-P. Brantut, J. Meineke, D. Stadler, S. Krinner, T. Esslinger, *Science* **337**, 1069 (2012).
10. D. Stadler, S. Krinner, J. Meineke, J. P. Brantut, T. Esslinger, *Nature* **491**, 736 (2012).
11. E. L. Hazlett, L.-C. Ha, C. Chin, arXiv:1306.4018.
12. G. D. Mahan, *J. Appl. Phys.* **65**, 1578 (1989).

10.1126/science.1245981

GENETICS

Genetics Driving Epigenetics

Terrence S. Furey^{1,2,3,4} and Praveen Sethupathy^{2,3,4}

Humans vary according to a plethora of traits, such as height, hair color, behavior, and susceptibility to disease. Both genetics (nature) and environment (nurture) contribute to this variation. Recent large-scale genetic studies have identified thousands of specific DNA variations in the human population that are associated with different traits. However, these studies do not answer a key question: By what means do most DNA variants alter cellular behavior and contribute to differences in specific traits, such as height? A trio of papers in this issue by Kasowski *et al.* on page 750 (1), Kilpinen *et al.* on page 744 (2), and McVicker *et al.* on page 747 (3) provide a framework for exploring the mechanistic link between genetic and trait variation in the human population. Specifically, they find that DNA variants influence a layer of gene regulation called epigenetics through the sequence-specific activity of transcription factors.

One of the most important discoveries in genetics in the last 10 years is that the vast majority of trait-associated DNA variations occur in regions of the genome that were once labeled as “junk DNA” because they do not code for proteins. We now know that these regions harbor genetic elements that control where, when, and to what extent specific genes are expressed to make functional RNA and protein products. Therefore, most trait-associated DNA variants are thought to alter not the gene itself, but rather, the regulatory elements that control the process of gene expression. In the last 3 years, several gene-regulatory variants have been strongly implicated in traits such as blood cholesterol concentrations (4) and diseases such as diabetes (5, 6), osteoarthritis (7), and prostate cancer (8). Despite these advances, precisely how most regulatory variants alter gene expression has been poorly understood.

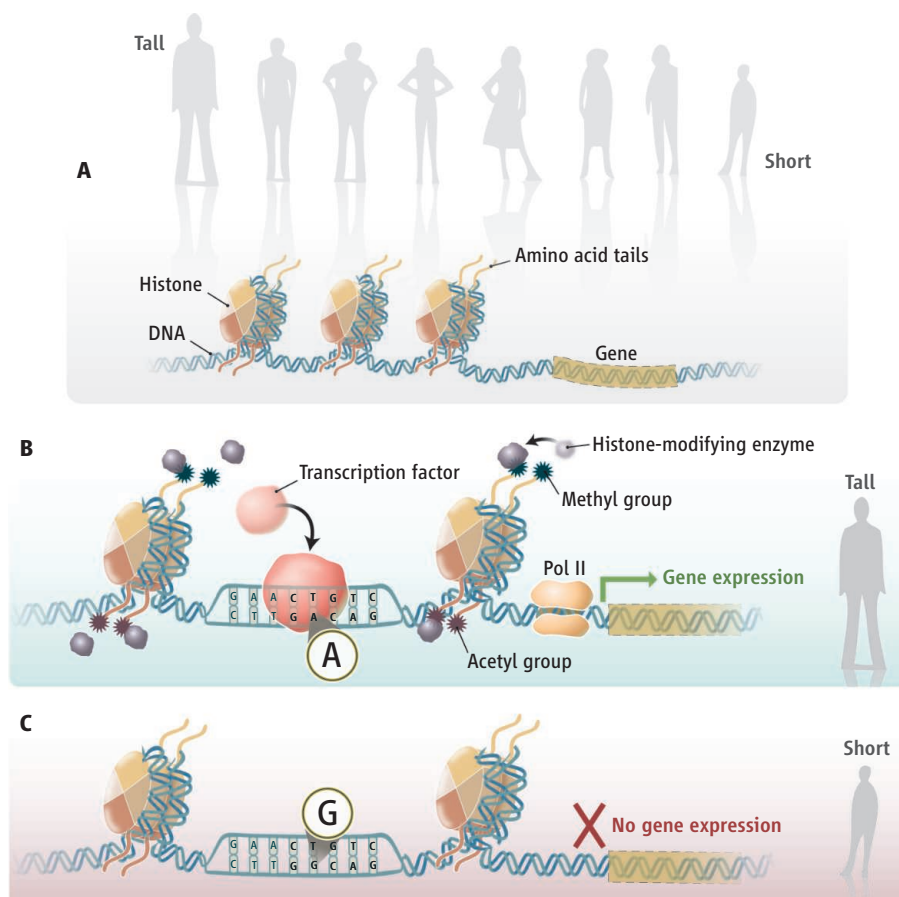
Epigenetic mechanisms are known to control heritable gene expression but have been generally viewed as independent of

the underlying DNA sequence. DNA is packaged in a three-dimensional structure, chromatin, whose basic repeating unit, the nucleosome, consists of ~146 nucleotides of DNA wrapped around an octamer of specialized proteins called histones. Each of the eight histone proteins has amino acid “tails” that stick out from the nucleosome. Specific amino acids in these tails are subject to a vast array of chemical modifications, such as methylation, acetylation, or phosphorylation, which are carried out by a variety of nuclear enzymes. The “histone code hypothesis” (9) proposes that specific combinations of histone tail modifications (epigenetic marks) are associated with transcription factors

DNA sequence variants are associated with factors and epigenetic states that modify gene expression and contribute to quantitative differences in traits.

that increase or decrease gene expression.

Kasowski *et al.*, Kilpinen *et al.*, and McVicker *et al.* perform integrative analysis of diverse data types generated from lymphoblastoid cell lines across numerous individuals and family trios. They demonstrate that histone tail modifications are highly variable in the human population and that they are heritable across generations. The studies identified hundreds of DNA variants that are associated with both histone tail modification and gene expression variation, indicating that genetics coordinates epigenetic effects on gene regulation. But how does variation in the DNA sequence influence chemical modification at histone tails?



Human trait variation. (A) Differences in a human trait (such as height) are partly due to the combined effects of genetic variants that alter the expression of multiple genes. (B) At a specific genomic position, a nucleotide [such as adenine (A)] is associated with accessible DNA, which facilitates transcription factor binding. This step leads to histone tail modifications that promote a chromatin environment favorable for the expression of neighboring genes. (C) At the same genomic position, a nucleotide variant [such as guanine (G)] has low affinity for transcription factor binding, which leads to a chromatin environment unfavorable for gene expression. Pol II, polymerase II

¹Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA. ²Department of Genetics, University of North Carolina, Chapel Hill, NC 27599, USA. ³Carolina Center for Genome Sciences, University of North Carolina, Chapel Hill, NC 27599, USA. ⁴Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA. E-mail: tsfurey@email.unc.edu; praveen_sethupathy@med.unc.edu

The three studies point to transcription factor activity as the missing link.

Most transcription factors bind directly to DNA, each with a preference for a particular DNA sequence pattern. Some DNA variants can substantially alter transcription factor binding affinity at particular genomic locations and thereby influence gene transcription. Kasowski *et al.*, Kilpinen *et al.*, and McVicker *et al.* used computationally predicted and empirically determined transcription factor binding data to identify hundreds of DNA variants that affect the strength of transcription factor binding. Many of these variants were also associated with variation in histone tail modifications.

These findings suggest a mechanism in which transcription factor binding to DNA initiates the recruitment of histone-modifying enzymes that set the histone tail modification pattern. Thus, a possible model (see the figure) is that trait-associated variants, most of which are gene regulatory in nature, affect the recruitment and binding of transcription factors to DNA. Differential transcription factor binding leads to variable histone tail modifications that collectively influence gene expression. Gene expression variations can manifest as trait differences.

Among some of the distinct findings of the studies, McVicker *et al.* showed that a single DNA variant can influence histone modifications at multiple related regions in the genome, providing information on their functional relationships. Kasowski *et al.* found that an individual's ancestry can affect what genomic regions exhibit genetically driven variability in chromatin marks. Kilpinen *et al.* noted that coordinated effects of DNA variation extend beyond transcription factor binding and histone tail modifications to other aspects of gene regulation, such as rate of transcription. Interestingly, all three studies found that many of the DNA variants associated with both transcription factor binding and histone tail modification variability were not associated with gene expression variability. This suggests that there is an abundance of nonconsequential regulatory variation, and/or that there are widespread mechanisms to compensate for the effects of regulatory variation, and/or that the regulatory effects of some transcription factor binding events are evident only under specific environmental conditions that are not well captured in cell culture.

The studies of Kasowski *et al.*, Kilpinen *et al.*, and McVicker *et al.* provide new insight into genetic mechanisms that affect

complex traits and disease, and also elucidate basic gene-regulatory processes, but by no means is either of these problems solved. For example, as the authors of these three studies emphasize, not every regulatory variant will lead to trait differences or even gene expression differences. Why are some regulatory variants more critical than others for trait variability or disease risk? There is much more to uncover to answer this and related questions, but these studies bring us one step closer and provide a framework for exploring this topic further.

References and Notes

1. M. Kasowski *et al.*, *Science* **342**, 750 (2013); 10.1126/science.1242510.
2. H. Kilpinen *et al.*, *Science* **342**, 744 (2013); 10.1126/science.1242463.
3. G. McVicker *et al.*, *Science* **342**, 747 (2013); 10.1126/science.1242429.
4. K. Musunuru *et al.*, *Nature* **466**, 714 (2010).
5. M. L. Stitzel *et al.*, *Cell Metab.* **12**, 443 (2010).
6. K. J. Gaulton *et al.*, *Nat. Genet.* **42**, 255 (2010).
7. A. W. Dodd, C. M. Syddall, J. Loughlin, *Eur. J. Hum. Genet.* **21**, 517 (2013).
8. M. M. Pomerantz *et al.*, *Nat. Genet.* **41**, 882 (2009).
9. B. D. Strahl, C. D. Allis, *Nature* **403**, 41 (2000).

Acknowledgments: We thank S. Kelada, M. Deshmukh, P. Rajasetupathy, M. Stitzel, and A. Laederach for critical reading and discussion.

10.1126/science.1246755

BIOCHEMISTRY

Have Your PIC!

Sohail Malik and Robert G. Roeder

RNA polymerase II (Pol II), the enzyme that transcribes protein-coding genes, requires additional factors to accurately initiate transcription from promoter-directed start sites. These general transcription factors (GTFs) assemble with Pol II into a pre-initiation complex (PIC) that is a key intermediate in the transcription activation pathway (1). The main role of the GTFs is to accurately position and orient Pol II on the DNA template and to facilitate access of the catalytic site to the transcribed strand. Recent structural studies, including the cryoelectron microscopy (cryo-EM) reconstruction of the yeast PIC by Murakami *et al.* on page 709 of this issue (2), provide detailed views into the structural organization of this large complex. These studies will pave the way for a more com-

plete understanding of how gene expression is regulated.

Biochemical studies have shown that the PIC initially forms on double-stranded promoter DNA (3). Prior to transcription of the body of the gene by Pol II, the PIC undergoes two critical transitions. First, it isomerizes into an open complex in which the promoter DNA exists in a partially melted state ("bubble") to provide the enzyme's active site access to the initiating nucleotide on the template. Next, in what is termed promoter escape, the melted region is extended in a discontinuous fashion to allow templated RNA chain synthesis to occur. This initially entails generation of short, abortive RNA products. Successful escape occurs when a stable RNA-DNA hybrid is established.

Murakami *et al.*'s structure shows that the PIC has two distinct lobes, reminiscent of the ribosome with which the PIC is often compared. The P-lobe is almost exclusively formed from Pol II subunits, whereas the

G-lobe comprises the various GTFs. Higher-resolution views reveal both the layout of individual subunits and the path of the template DNA, which, while sandwiched between the two lobes, is mainly associated with the G-lobe. This arrangement is surprising; one might have expected from earlier biochemical studies that the DNA, Pol II, and GTFs would be intertwined in the PIC.

The GTFs include TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIH (see the figure, panel A). Consistent with previous studies (4–6), Murakami *et al.*'s structure (see the figure, panel B) shows how these individual GTFs are arrayed with respect to the template and Pol II, poised for their roles in the various transitions that the PIC will undergo. Murakami *et al.* do not present a structure for the open state, but their PIC reconstruction suggests hypotheses for how Pol II engages the template strand after transfer of the DNA from the G-lobe to the Pol II cleft, at the bottom of which lies the catalytic cen-

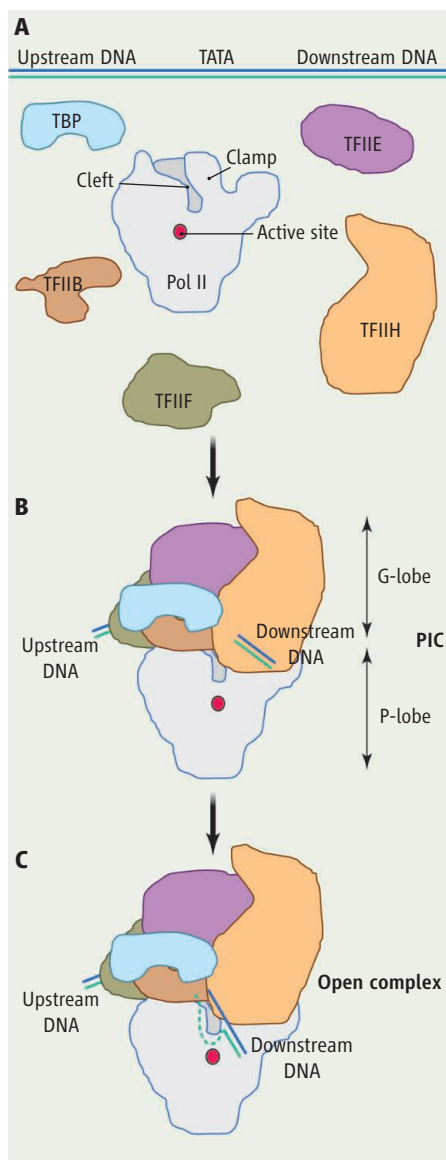
Laboratory of Biochemistry and Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10065, USA.
E-mail: maliks@rockefeller.edu; roeder@rockefeller.edu

ter (see the figure, panel C). Based on previous studies (5, 6), TFIID might contribute to open complex formation either through a torquing or pumping action on DNA. He *et al.* recently reported an EM structure for an open-complex mimic formed by human Pol II and GTFs (7). However, because of technical limitations, the precise roles of TFIID in the closed-to-open transition and the potentially changing path of downstream DNA are not readily evident even from that structure.

Several recent crystal structures have further elucidated dynamic aspects of PIC function that relate to template melting and initial RNA synthesis. A structure of Pol II in association with TFIIB and a DNA-RNA scaffold mimicking the open complex reported by Sainsbury *et al.* (8) shows how this GTF fulfills critical roles both in promoter melting and in ensuring an orderly transition into the productive phase of transcription by overseeing the process of promoter escape. TFIIB achieves this in large part through multivalent interactions between discrete domains within its N terminus and Pol II, DNA, and the nascent RNA.

Together, these structural studies show that the PIC is essentially a device for overcoming constraints inherent to the transcription process. Bending and melting mechanisms have evolved as direct responses to template rigidity. Furthermore, and in contrast to DNA polymerases, the relatively involved mechanism for Pol II likely reflects the fact that it commences initiation without a preformed primer. Also unlike DNA polymerases, Pol II needs to separate its product (RNA) from the template strand. It is remarkable that all three eukaryotic RNA polymerases are homologous not only in their basic subunit composition but also with respect to their dedicated initiation factors (9). Thus, the fundamental mechanisms being uncovered by these studies would be equally applicable to Pol I and Pol III.

Complementing these structural studies, which can interrogate only some of the PIC's many dynamic states, biophysical studies have attempted to catch the PIC in action. Treutlein *et al.*'s nano-positioning system analysis, in which fluorescent FRET probes were attached to the template, Pol II, or TFIIB, has served as a molecular ruler to measure distances between key PIC landmarks, which in turn enables monitoring of changes in the PIC during key transitions such as promoter escape (10). Single-molecule methods, in which function can be monitored by fluorescence video microscopy of surface-immobilized and derivatized PICs,



How transcription begins. (A) Pol II and the GTFs assemble on DNA templates that contain promoter elements such as the TATA box, which is recognized by the TBP subunit of the TFIID complex. (B) The PIC structure reported by Murakami *et al.* suggests that cooperative interactions among Pol II, GTFs, and DNA are a major driving force for its formation. (C) Details of the open promoter complex are sketchy, but DNA movement toward the cleft and descent of the separated template strand into the active site have been predicted (7, 8). TFIIA has been omitted for clarity.

have also highlighted the stochastic nature of the transcription process (11).

Much of the current understanding of PIC structure and function has necessarily come from reductionist approaches, and much remains to be learned about the functioning of even a minimal PIC. However, we must not lose sight of the biological context in which the PIC exists. A PIC is typically formed in response to gene- and cell-specific DNA

binding transcriptional activators that in turn rely on coactivators, notably the Mediator complex, to transduce signals to the PIC (12). The Mediator is in intimate contact with PIC components and might even be regarded as a GTF in its own right. Other factors include the TAF subunits of TFIID (which have not been included in structural analyses of reconstituted PICs) and the SAGA complex.

Furthermore, the initiation factors discussed here compete with elongation factors that bind Pol II as it escapes the promoter (13). This is particularly an issue in metazoan cells, where promoter-proximal pausing of Pol II is widespread with important implications for how PIC function is affected (14). Superimposed on these interactions is the effect of the chromatin environment of the PIC. In yeast cells, the +1 nucleosome seems to dictate precisely what kind of a PIC (TAF-containing or SAGA-containing) will assemble in a given nucleosome context (15).

Future structural studies will no doubt aim to incorporate these additional layers of complexity into our PIC framework, but at least one approach—in which structures of PICs assembled from unfractionated cell extracts are probed by chemical cross-linkers—has been yielding reliable readouts of various GTF locations in a more natural context (6). It is anticipated that cross-linking and mass spectroscopy (XL-MS) technologies, such as the one used by Murakami *et al.*, will be further developed to probe the PIC in its various guises. Equally promising, newly developed genome-wide techniques to probe the PIC at close to single-nucleotide resolution (15) should provide glimpses into the dynamics of the PIC in living cells.

References and Notes

1. R. G. Roeder, *Trends Biochem. Sci.* **21**, 327 (1996).
2. K. Murakami *et al.*, *Science* **342**, 1238724 (2013).
3. S. Hahn, *Nat. Struct. Mol. Biol.* **11**, 394 (2004).
4. D. B. Nikolov *et al.*, *Nature* **360**, 40 (1992).
5. T. K. Kim, R. H. Ebright, D. Reinberg, *Science* **288**, 1418 (2000).
6. S. Grünberg, L. Warfield, S. Hahn, *Nat. Struct. Mol. Biol.* **19**, 788 (2012).
7. Y. He, J. Fang, D. J. Taatjes, E. Nogales, *Nature* **495**, 481 (2013).
8. S. Sainsbury, J. Niesser, P. Cramer, *Nature* **493**, 437 (2013).
9. A. Vannini, P. Cramer, *Mol. Cell* **45**, 439 (2012).
10. B. Treutlein *et al.*, *Mol. Cell* **46**, 136 (2012).
11. A. Revyakin *et al.*, *Genes Dev.* **26**, 1691 (2012).
12. S. Malik, R. G. Roeder, *Nat. Rev. Genet.* **11**, 761 (2010).
13. S. Malik, M. J. Barrero, T. Jones, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 6182 (2007).
14. A. H. Ehrensberger, G. P. Kelly, J. Q. Svejstrup, *Cell* **154**, 713 (2013).
15. H. S. Rhee, B. F. Pugh, *Nature* **483**, 295 (2012).

Acknowledgments: This work was supported in part by U.S. Department of Defense grant BC121740.

10.1126/science.1246170

Epithelial Plasticity: A Common Theme in Embryonic and Cancer Cells

M. Angela Nieto

Background: During embryonic development, cells often travel long distances to form tissues and organs. To be able to migrate, embryonic cells undergo a process known as epithelial-to-mesenchymal transition (EMT). Once migratory embryonic cells reach their destination, they undergo the reverse process, mesenchymal-to-epithelial transition (MET), to later differentiate into multiple cell types. This reveals a high degree of cell plasticity, referring to the ability of cells to reversibly change phenotype, a common feature of embryonic cells. Research indicates that the EMT program is reactivated in cancer cells in the delamination from a primary tumor, the first step toward the colonization of distant organs to form secondary tumors (metastasis). The dissemination of cancer cells and the subsequent formation of metastasis are responsible for the vast majority of cancer-associated deaths. As in EMT, recent advances show that cancer cells rely on the reactivation of developmental programs through MET for the localization and proliferation of disseminating cells. The embryo provides clues to understanding the complex cell biology of EMT and MET in cancer and moving toward improved therapeutic strategies.

Advances: Due to the importance of the EMT and MET programs in normal development for the generation of tissues and organs, as well as their role in cancer, stringent regulatory mechanisms are needed. Multiple extracellular signals converge in the activation of transcription factors that can trigger the full EMT program. In addition, epigenetic and splicing programs, as well as microRNA regulatory networks, control epithelial plasticity toward EMT or MET. As differentiated normal and cancer cells can reenter an undifferentiated stemlike state, another level of cell plasticity has become apparent, helping to elucidate complex cell behaviors and interactions.

Outlook: Technical advances in noninvasive in vivo imaging of embryos will help define cell behavior and plasticity in normal development, fundamental to the understanding of congenital malformations. This knowledge will undoubtedly facilitate the study of tumor progression in animal models of cancer. Cancer cells can also be directly interrogated about their plastic states in molecular terms after the purification and analysis of circulating or disseminated single cells from animal models and also from patients, aiding in the design of improved therapies. With respect to antimetastatic therapies, inhibiting EMT may be counterproductive in tumors that disseminate early, as rather than preventing metastasis, it could favor the formation of secondary tumors from already disseminated cells. Strategies aimed at targeting cancer stem cells are very promising, but it is important to consider that new cancer stem cells can be produced from differentiated nonstem bulk tumor cells.

READ THE FULL ARTICLE ONLINE

<http://dx.doi.org/10.1126/science.1234850>



Cite this article as M. A. Nieto, *Science* **342**, 1234850 (2013). DOI: 10.1126/science.1234850

ARTICLE OUTLINE

Global Cellular Programs Regulate Epithelial Plasticity

EMT in Embryonic Development and the Delamination of Cancer Cells from Primary Tumors

Reversal of Developmental EMTs for Organ Formation

Reversal of EMTs for Metastatic Colonization

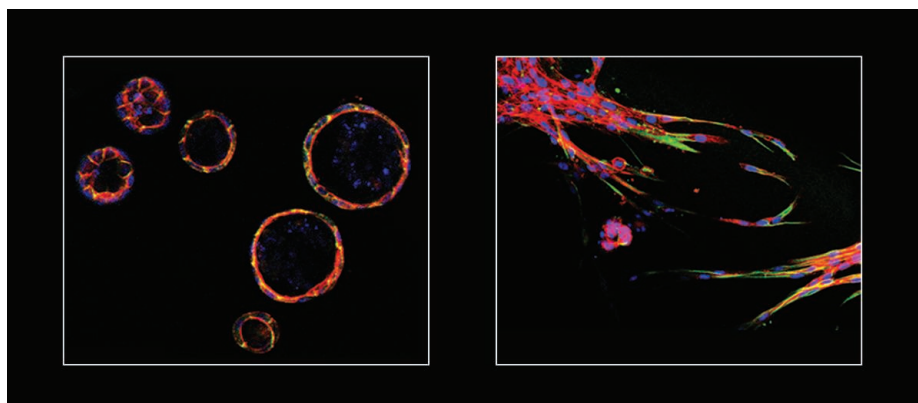
Uncoupling EMT and Stemness

EMT and MET in Cancer: Conflicting Data or Just Plasticity?

Toward Improved Metastatic Therapeutic Strategies

SUPPLEMENTARY MATERIALS

Movies S1 and S2



Epithelial plasticity in three-dimensional (3D) cultures. Epithelial cells [Madin-Darby canine kidney (MDCK) cells] form ducts when grown on 3D matrices resembling the in vivo microenvironment (**left**). When grown under identical conditions, MDCK cells expressing Prrx1 (an EMT inducer) form networks of mesenchymal cells (**right**). Note the dramatic phenotypic change that is accompanied by the acquisition of motility and invasive properties. Blue, nuclei revealed by 4',6-diamidino-2-phenylindole staining; red, actin filaments as seen after phalloidin binding; green, E-cadherin (epithelial marker) (**left**) and vimentin (mesenchymal marker) (**right**).

Epithelial Plasticity: A Common Theme in Embryonic and Cancer Cells

M. Angela Nieto

During embryonic development, many cells are born far from their final destination and must travel long distances. To become motile and invasive, embryonic epithelial cells undergo a process of mesenchymal conversion known as epithelial-to-mesenchymal transition (EMT). Likewise, EMT can be seen in cancer cells as they leave the primary tumor and disseminate to other parts of the body to colonize distant organs and form metastases. In addition, through the reverse process (mesenchymal-to-epithelial transition), both normal and carcinoma cells revert to the epithelial phenotype to, respectively, differentiate into organs or form secondary tumors. The parallels in phenotypic plasticity in normal morphogenesis and cancer highlight the importance of studying the embryo to understand tumor progression and to aid in the design of improved therapeutic strategies.

Cellular plasticity refers to the ability of cells to reversibly change their phenotype. An example is seen in early metazoan embryogenesis, where epithelial cells take on mesenchymal characteristics, a process termed epithelial-to-mesenchymal transition (EMT). The EMT implies the conversion of an epithelial cell into a mesenchymal cell with the ability to migrate and invade adjacent tissues. Through the associated changes in adhesion and behavior, cells can move into the interior of the embryo, travel long distances, and participate in the formation of internal organs (1). Importantly, the latter implies the reversibility of the EMT, as when cells arrive to their destination they usually revert to the epithelial phenotype, undergoing a mesenchymal-to-epithelial transition (MET) to settle, proliferate, and differentiate into different organs (2) (Fig. 1).

The hallmarks of the EMT can be summarized as follows: loss of cell-cell junctions, loss of apico-basal polarity, and acquisition of migratory and invasive properties (3) (Fig. 1). This phenotypic change is accompanied by a dramatic change in cell behavior (see supplementary movies) and is triggered in response to extracellular signals by the activation of one or several transcription factors (TFs) belonging to different families, including Snail, Twist, Zeb, and others (4), which are generally termed EMT-TFs (5). These EMT-TFs elicit the EMT program by repressing the epithelial phenotype, enhancing mesenchymal traits including motility, and inducing the ability to degrade the basement membrane and the extracellular matrix in general. In addition, EMT-TFs also impinge into other basic cellular processes that help maintain the mesenchymal phenotype, the efficacy of migration, and survival in adverse

environments. As such, EMT-TFs attenuate proliferation and protect from cell death—activating survival signaling cascades (2) (Fig. 1). Due to the pivotal role of E-cadherin loss, EMT-TFs are sometimes referred to as E-cadherin repressors; also, a decrease in functional E-cadherin is of-

ten associated with the activation of EMT. However, it is important to bear in mind that the EMT program and EMT-TFs are much more than E-cadherin transcriptional repressors, that E-cadherin down-regulation (or endocytosis) is not necessarily associated with the EMT program, and that E-cadherin reexpression is not sufficient to revert the fibroblastic phenotype (6).

Cells that escape from carcinomas and are converted into mesenchymal cells with migratory and invasive properties have undergone an EMT (2, 3), and many EMT-TFs have been characterized that operate during tumor progression (7). However, the relevance of the EMT in cancer biology has been a matter of debate (8). The application of sophisticated imaging techniques to animal models and the characterization of circulating tumor cells (CTCs) from patients as cells with an EMT signature have provided evidence that the EMT also occurs during the dissemination of cells from a primary tumor (9–12).

Because the EMT can also endow cells with stem cell properties, and given that cancer can be initiated, maintained, and propagated by stationary and motile cancer stem cells (CSCs) (5, 13), a new field of study has emerged that requires the concerted effort of developmental biologists and

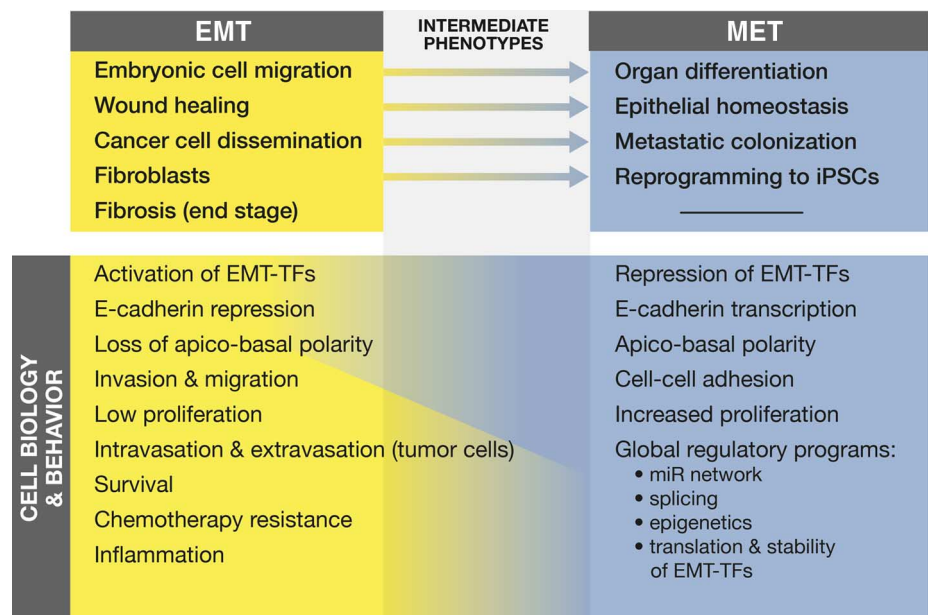


Fig. 1. The control of epithelial plasticity in development and disease. Embryonic epithelial cells undergo EMT to migrate and then revert to the epithelial phenotype at their destination to give rise to different tissues and organs. After injury, epithelial cells undergo a partial EMT to heal the wound. Later, these cells undergo a MET to maintain epithelial homeostasis. Likewise, disseminated cancer cells need to return to the epithelial state during metastatic colonization, accompanied by the recovery of a high proliferation potential for the establishment of a secondary tumor. A MET is also required during reprogramming of fibroblasts to iPSCs. The development of organ fibrosis also involves EMT, which does not revert and leads to organ degeneration and failure. Both developmental and pathological EMTs not only induce morphological changes but also provide motility and invasive properties together with the ability to overcome safe-guard mechanisms, leading to cell survival and chemotherapy resistance, which is particularly relevant in cancer patients. Global cellular programs—including epithelial-specific splicing, epigenetic mechanisms, and miRNA regulatory networks—are in place to protect epithelial homeostasis.

cancer researchers. However, even though developmental and pathological EMTs have many common features, they also have obvious differences, leading to the classification of three different EMT types (14). Type 1 refers to developmental EMTs; type 2 denotes those related to wound healing, tissue regeneration, and organ fibrosis; and type 3 indicates the EMT associated with cancer. Developmental EMTs imply the conversion of epithelial into mesenchymal cells, but embryos lack inflammatory responses, typical of type 2 and 3 EMTs that occur in the adult (14, 15). Type 2 EMTs are also characterized by the appearance of myofibroblasts with the ability to secrete an excess of extracellular matrix molecules in response to inflammation (14). In contrast to the situation in embryos, in cancer cells and during regeneration, the acquisition of a mesenchymal state in fibrosis can be considered an end stage, which leads to organ degeneration and failure (Fig. 1). During cancer progression, tumor cells undergo type 3 EMT, which, in addition to invasion and motility, involves intravasation of delaminated cells into lymphatic and blood vessels and their subsequent extravasation. The intravasation and extravasation steps do not occur in fibrosis and do not have obvious parallels with the migratory processes undergone by embryonic cells. In this Review, I will focus on the similarities—in particular, between embryonic and cancer cells—and how the latter rely on developmental programs, not only to delaminate from the initial tumor but also to later colonize distant sites. The common theme of epithelial cell plasticity unifies both events.

Global Cellular Programs Regulate Epithelial Plasticity

The EMT is triggered by many extracellular signals and agents in both embryos and cancer cells. The most potent inducers are members of the transforming growth factor- β (TGF β)/bone mor-

phogenetic protein (BMP) family, but also Wnt, Notch, epidermal growth factor (EGF), fibroblast growth factor, hypoxia, obesity, alcohol, nicotine, ultraviolet light, and others [reviewed in (2)]. Such pathways and agents may act alone or in combination, and the response is dependent on the cell context (2). The response to such signals converges on the activation of the EMT-TFs, which can cooperate to induce or maintain the mesenchymal phenotype (4, 16). The existence of many EMT-TFs provides robustness to the system, ensuring the implementation of the EMT program during embryonic development (1, 17). Analyzing regions where EMT is induced in embryos indicates that Snail expression normally precedes the expression of other factors, which are more relevant for the maintenance of the mesenchymal phenotype (2, 4). This temporal hierarchy of EMT-TF activation and cooperation also seems to operate in tumors, as observed for Snail factors and Twist in breast epithelial cells and breast cancer progression (16, 18–20). Once activated, EMT-TFs can, in turn, activate the same signaling pathways to generate positive regulatory autocrine loops that help maintain the mesenchymal phenotype both in normal and transformed breast stem cells (21).

Because EMT may be extremely deleterious when aberrantly activated in the adult (3), it needs to be very tightly regulated to ensure epithelial homeostasis. Besides the transcriptional level, there is also posttranscriptional, posttranslational, and epigenetic control, including a splicing program specifically associated with the epithelial phenotype and regulation by noncoding RNAs [recently reviewed in (22–24)] (Fig. 1). The epigenetic, splicing, and microRNA (miRNA) networks operating during EMT are now being defined at the whole-genome level (25–28), identifying global regulatory programs that control the EMT. Such programs provide specificity and robustness through their phenotypic impact on

each aspect of the EMT in both embryos and cancer cells [reviewed in (17, 24)] (Fig. 1).

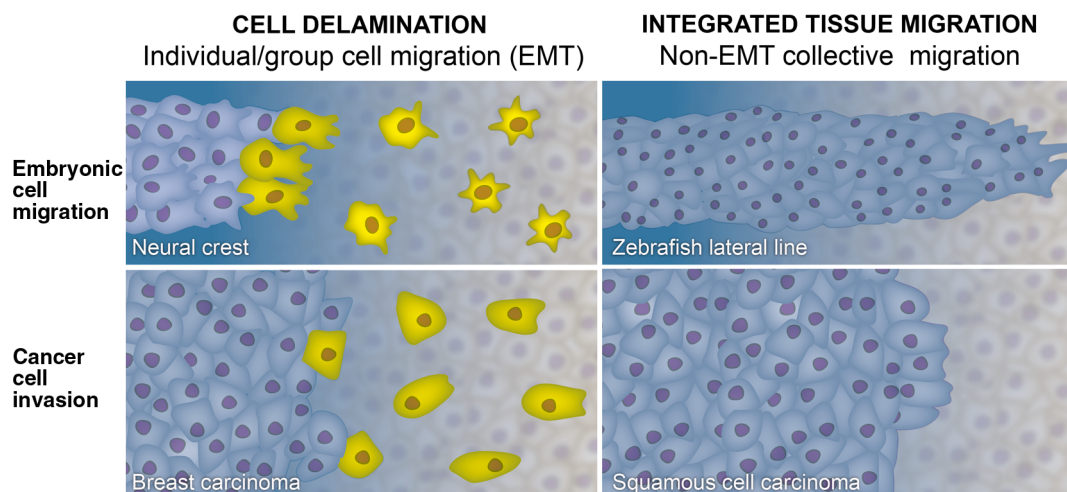
EMT in Embryonic Development and the Delamination of Cancer Cells from Primary Tumors

The internalization of cells from the surface of the embryo to form organs often involves an EMT. Cells disrupt cell-cell adhesion contacts within the primitive epithelial layer and ingress. The best-studied examples are the ingression of mesendodermal precursors at the primitive streak in amniotes and the delamination of the neural crest (Fig. 2). However, it is worth noting that many additional EMT processes exist in the embryo, as with the exception of the anterior central nervous system and the epidermis, all vertebrate adult tissues originate from cells that underwent at least one EMT. The best evidence of EMT in cancer comes from the observation of single cells delaminating from primary tumors (9) and from the finding that CTCs and disseminated tumor cells (DTCs) from patients show EMT features and a high degree of epithelial plasticity (10, 11) (Fig. 2).

In development and disease, EMT is not necessarily an “all-or-nothing” transition. Partial EMTs, in which cells share epithelial and mesenchymal traits, can occur. Partial EMTs occur during *Drosophila* gastrulation and during the early migration of groups of neural crest cells in anamniotes (amphibian and fish). These cell movements are sometimes referred to as collective migration to indicate that cells move in a coordinated manner while maintaining some cell-cell contacts (29). However, in terms of molecular mechanisms, cells have engaged into the EMT program during these processes, down-regulating *E-cadherin* transcription and losing apico-basal polarity upon activation of EMT-TFs (3). Partial EMTs are also observed in the epithelial component of carcinosarcomas (30) and at intermediate states during

Fig. 2. Cell migration in embryos and tumors.

Individual migration occurs after epithelial cells undergo EMT, as for neural crest migration during normal embryonic development and the delamination of breast cancer cells from the primary tumor. Partial EMT can also occur when cells activate the EMT program but maintain some contacts with their migrating neighbors. Integrated tissue migration implies a decrease in cell-cell adhesion forces but the maintenance of epithelial traits, including cadherin expression and apico-basal polarity. This type of migration is observed in the developing zebrafish lateral line and in the invasive front of some carcinomas. Cells at the invasive front can also activate the EMT program, in which case they can be seen delaminating as individual or loosely organized groups of cells. Blue, epithelial cells; yellow, mesenchymal cells (after EMT).



the progression of organ fibrosis, when parenchymal (epithelial) cells have activated the EMT program and show both epithelial and mesenchymal markers (37).

Cell movements can also occur in the absence of an EMT, when cells move while maintaining epithelial integrity. Examples of this type of movement include the migration of the lateral line primordium in the zebrafish embryo (Fig. 2) and that of border cells in the *Drosophila* egg chamber (32). These cells do not express EMT-TFs, maintain cell junctions (including E-cadherin expression), and normally also maintain apico-basal polarity. These movements can also occur in cancer, when tumor cells invade without segregating from the invasive front (Fig. 2), and are able to colonize lymph nodes. However, in contrast to cancer cells that have undergone an EMT, cells that have not undergone an EMT are not able to intravasate into blood vessels to later develop blood-borne distant metastasis (33).

Reversal of Developmental EMTs for Organ Formation

As noted above, developmental EMTs are reversible (34). As such, the cells that go through so called primary EMTs in the embryo later undergo the reverse process, MET, to differentiate into multiple cell types. Early mesodermal cells, generated by EMT and located at different mediolateral lev-

els of the embryo (axial, paraxial, intermediate, and lateral plate mesoderms), condense into transient epithelial structures through MET to give rise to the notochord, the somites, the primordia of the urogenital system, and the splanchnopleura and somatopleura, respectively (Fig. 3). In the majority of these structures, a second round of EMT is completed. For example, the dorsal somite converts into the dermal and myotomal mesenchyme, which will give rise to components of the dermis and the muscle satellite cells. The ventral part will become the mesenchymal sclerotome that later generates the vertebrae, the ribs, and the tendons (Fig. 3). In turn, precursor cells of endodermal organs, such as the pancreatic endocrine cells, migrate upon undergoing EMT and revert this migratory phenotype through a MET to form the Langerhans islets. A notable example of epithelial plasticity is the development of the cardiac valves, for which the initial mesenchymal precursors are specified at gastrulation stages and must undergo three consecutive rounds of EMT and MET (2).

Although many signaling pathways have been identified for EMT (2, 24), the signals involved in the induction of MET have not been well characterized in embryonic development. The most studied and amenable processes are those associated with the epithelialization of the paraxial and intermediate mesoderm to form the somites and the precursors of the renal system, respec-

tively. An increasing gradient of BMP signaling along the mediolateral axis patterns the different mesoderms, with lower concentration specifying the paraxial mesoderm. BMP7 counteracts TGF β signaling and is the most prominent known epithelializing agent, with examples seen during kidney development (35), during reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) (36), in mouse models of renal fibrosis (37), and in cancer cells (38). Eph and Ephrin signaling is also involved in the epithelialization of somites (39), and Wnt signaling is required for MET during nephron development (40).

For nearly all MET processes, there is a concomitant down-regulation of the corresponding EMT-TFs [i.e., (41, 42)]. This down-regulation is accompanied by the appearance of specific epithelial markers that are associated with differentiation into the distinct tissues and organs. In the case of the epithelialization of the somitic and pronephric tissues, MET is associated with the induction of Paraxis and Pax2, respectively.

Reversal of EMTs for Metastatic Colonization

Despite the observations above, it is not universally accepted that tumor cell dissemination is related to a change in cell identity [a requirement for EMT, see (8)]; rather, this process might rely on mutations that weaken the cell-cell adhesion of cancer cells. Nevertheless, it is clear that carcinoma metastases usually adopt a well-differentiated epithelial phenotype. Ironically, this is one of the arguments used to dismiss the importance of the EMT in cancer progression. However, the differentiated phenotype of metastases reflects the epithelial plasticity implicated in tumor progression—in other words, the reversion of EMT first suggested by Brabletz *et al.* (43) and again resembling the situations described during embryonic development (34).

As in the embryo, MET concurs with the down-regulation of EMT-TFs in cancer cells. MET not only implies a reversion to the epithelial phenotype but also an increase in cell proliferation, important for the growth of the secondary tumor. Several transcriptional repressors that can repress EMT-TFs have recently been identified, including Kruppel-like factor 17 (KLF17) (44), E74-like factor 5 (ELF5), (45) and Single-minded 2s (SIM2s) (46). Both ELF5 and SIM2s repress *Snail2* expression. Conversely, *Snail* represses *Single-minded* during *Drosophila* gastrulation (47), likely revealing an additional regulatory loop in the control of epithelial plasticity.

Complex regulatory loops also control epithelial plasticity posttranscriptionally, as numerous miRNAs are integrated into EMT and MET regulatory networks (22, 24). Double-negative feedback loops have been described between EMT-TFs (Snail, Zeb, and Gata3 factors) and several miRNAs, including members of the miRNA-200 (miR-200) family and miR-34a (48–50), which promote MET and protect the epithelial phenotype (Fig. 4). Recently, an additional miRNA has been added to this network, as miR-22 promotes EMT and cancer progression by indirectly repressing miR-

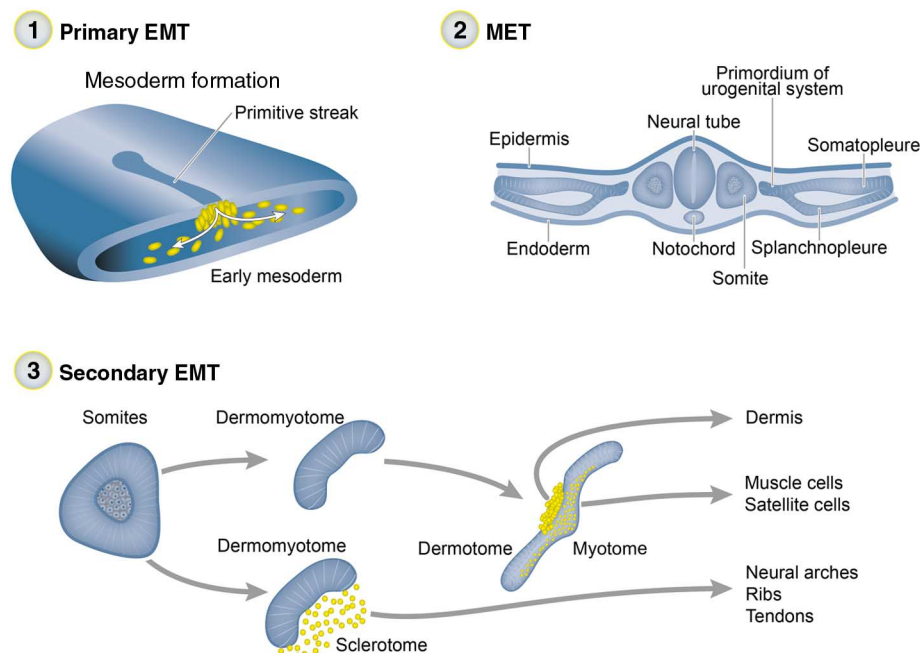


Fig. 3. Reversibility of EMT during embryonic development. Physiological EMTs are reversible. With the exception of the epidermis and part of the central nervous system, adult vertebrate tissues are the result of one or several rounds of EMT and MET. This figure follows a temporal developmental sequence: (1) The precursors of the endoderm and the mesoderm ingress at the primitive streak, a prototypical example of primary EMT. (2) The mesodermal precursors migrate to occupy different positions along the mediolateral axis of the embryo to give rise to axial, paraxial, intermediate, and lateral mesoderm, which upon undergoing MET will generate notochord, somites, the urogenital system, and the splanchnopleura and somatopleura, respectively. (3) The majority of these epithelial derivatives undergo a second round of EMT. The figure exemplifies secondary EMTs, showing those occurring in the somites (see text). Blue, epithelial cells; yellow, mesenchymal cells after EMT.

200 (51), and miR-506 has been identified as a node that promotes MET (28) (Fig. 4).

Although TGF β is a potent inducer of EMT-TFs, the tumor suppressor p53 activates miR-34a and miR-200 (50, 52) (Fig. 4). The latter can be antagonized by the opposing role of mutant p53, which activates Zeb1 through the repression of miR-130 (53). Another p53 family member, p73, and its truncated antagonistic form DNp73, also play opposing roles in tumor progression: Whereas p73 behaves as a tumor suppressor, DNp73 induces EMT (54). Going back to the aforementioned miRNAs, though much less is known about their function during embryonic development, it is clear that miRs help the establishment of embryonic territories (55) and that a double-negative regulatory loop is established between miR-200 and Sox2 transcription factor during neuronal differentiation (56). The expression pattern of miR-200 in developing embryos is compatible with its role in preserving the epithelial phenotype and promoting MET.

Induction of MET by miR-200 was shown to promote breast cancer metastasis (57), in agreement with earlier findings indicating that MET facilitates bladder cancer metastasis (58) and the observation that myeloid cells induce a MET in the metastatic niche (59). However, until very recently, a requirement for epithelial plasticity and the reversion of EMT had yet to be directly assessed in vivo.

In their 2012 study, Tsai *et al.* used a spontaneous squamous cell carcinoma model in mice carrying skin-specific inducible Twist to show that Twist-mediated EMT is sufficient to promote the dissemination of cancer cells into the bloodstream. Yet more importantly, Tsai *et al.* used this model to show that Twist needs to be down-regulated for metastasis to occur (60). Further in vivo evidence for epithelial plasticity and the requirement of MET has been found in the progression of breast cancer to the metastatic state. EMT mediated by an EMT-TF, the paired-related homeobox 1 (Prrx1 factor) needs to be reverted, and Prrx1 loss is required for metastatic colonization (16). Therefore, a reversible EMT seems to be necessary for the metastasis of primary carcinomas, revealing a developmental program that is reactivated by cancer cells for them to successfully complete the final step of the metastatic cascade, the formation of macrometastasis.

Uncoupling EMT and Stemness

EMT can confer stemlike properties on cells (61, 62). This is consistent with the concept of “migratory cancer stem cells” (63) and provides a link between the EMT program and the characteristics associated with CSCs; that is, self-renewal and the capacity to seed secondary tumors and produce differentiated nonstem cells (5). However, the relation between EMT and stemness is another controversial issue in tumorigenesis, as it was later shown that fibroblasts must undergo a MET to complete the initial reprogramming of fibroblasts to iPSCs (36, 64). Again, help may

come from development, although CSCs differ from canonical embryonic stem cells (ESCs) in that they revert to the phenotypes of only the primary tumor and, therefore, have a more restricted potency than both ESCs and iPSCs (65).

The need for a MET during reprogramming is consistent with the epithelial nature of ESCs. In fact, the Yamanaka factors (Sox2, Klf4, Oct4, and Myc) act together with BMP7 to repress TGF β signaling and EMT-TFs to revert fibroblasts to the epithelial phenotype (64). In agreement with this, both Snail1 and Prrx1 are down-regulated during reprogramming before cells acquire pluripotency genes (66). In line with the incompatibility of a stable EMT phenotype and metastatic colonization, Celià-Terrassa *et al.* (67) have shown that EMT can suppress tumor-initiating capacities (TICs) in epithelial cells. Together, these results link the MET with stemlike properties, rather than EMT with stemness as proposed previously. A plausible explanation for this apparent contradictory data may be that epithelial plasticity and stemness are regulated independently. The MET undertaken during reprogramming of fibroblasts requires continuous presence of the Yamanaka

factors and does not initially involve the acquisition of stemness, which represents a later event (36). In addition, one of the EMT-TFs, Snail2, cooperates with Sox9 in the acquisition of the mesenchymal mammary stem cell state, although it seems that Snail2 is mainly involved in the induction of EMT, and Sox9 is responsible for the entry into the stem cell state (68). Further evidence of such independent regulation comes from the analysis of Prrx1, which, unlike classical EMT-TFs (Snail, Twist, and Zeb factors), induces EMT without concomitantly inducing CSC properties. By contrast, the loss of Prrx1 facilitates self-renewal and mammosphere-forming ability in breast cancer cells (16), simultaneously inducing MET and TIC, both favoring metastatic colonization.

How Prrx1 down-regulation is achieved at the metastatic niche and how TIC is maintained upon the down-regulation of EMT-TFs linked to stemness (Snail-type) is a matter of future investigation (Fig. 5). Another pending issue is whether the two EMT types are parallel or exclusive processes in a particular tumor. On the one hand, Prrx and Snail do not show any correlation in data sets from patients. However, Prrx1 and Twist

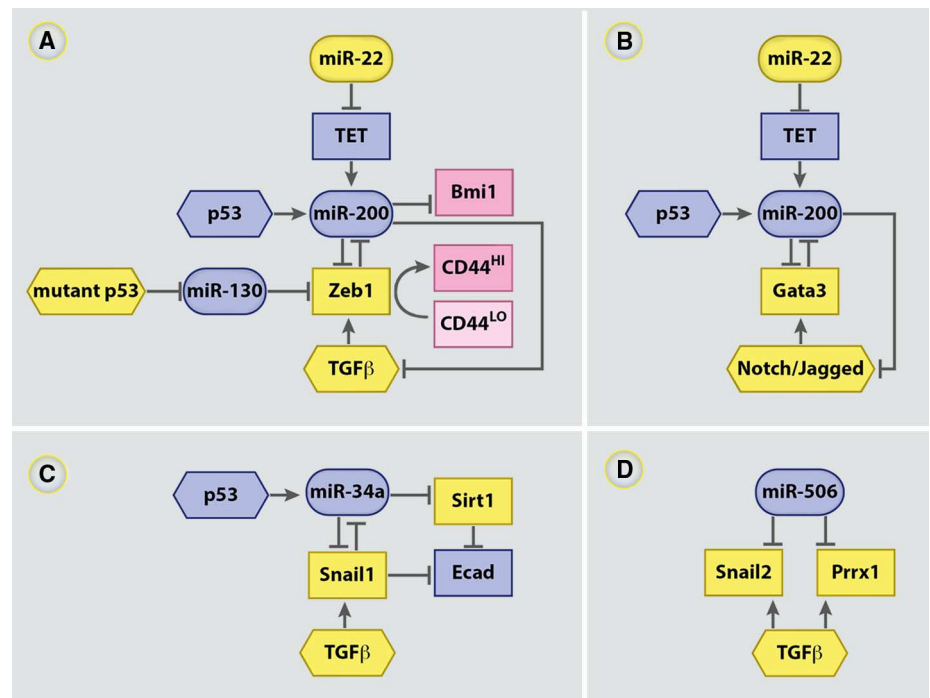


Fig. 4. Epithelial plasticity and miRNA regulatory networks. (A to D) Signaling pathways mediated by TGF β or the Notch ligand Jagged induce the expression of EMT-TFs (Zeb, Snail, Prrx, or Gata3 factors), which are down-regulated by a series of miRNAs (miR-200, miR-130, miR-34a, and miR-506) to protect epithelial integrity. Double-negative feedback loops are established in which miRNAs and the TFs behave as reciprocal repressors. The tumor suppressor p53 prevents EMT by activating miR-200 and miR-34a. By contrast, mutant p53 and miR-22 activate EMT by repressing miR-130 and miR-200, respectively. Epigenetic regulation of the *Zeb1* and *miR-200* promoters impinges into their feedback loop. The bivalent state of the *Zeb* promoter allows its rapid activation, EMT, and reentry into the CSC state by increasing the expression of the surface marker CD44. By contrast, the TET complex demethylates the *miR-200* promoter, thereby favoring MET. Sirtuin 1 (Sirt1) favors EMT by cooperating with EMT-TFs in the repression of E-cadherin expression. miR-506 is a newly described EMT regulator able to repress several EMT-TFs, including Snail2 and Prrx1. Blue, MET related molecules; yellow, EMT related molecules; pink, CSC markers.

are correlated, and in these cases, Prrx1 prevails (16), as PRRX1 down-regulation is sufficient to induce MET and CSC properties. It is possible that both types (Snail and Prrx) could coexist, as depicted in Fig. 5, but information is still lacking. On the other hand, isolated CTCs depict both EMT and CSC traits (10, 11), suggesting that they might not include the Prrx1 type. This has not been specifically addressed, but it is likely that in purified CTCs, Prrx1-positive cells are underrepresented, as they are fully mesenchymal and might have escaped isolation with current purification protocols, which use epithelial markers to identify CTCs (10, 11).

Data accumulated over the past few years indicate that EMT-TFs are developmental factors that, when aberrantly activated in tumors, initiate the invasion-metastatic cascade. This finding also favors the cancer stem cell model over the clonal evolution model by which successive mutations accumulate in a tumor, providing a defined phenotype (69). However, two additional issues need to be discussed here: On the one hand, stemness is also a plastic state during tumor progression, whereby both stationary and migratory CSCs can coexist (13). On the other hand, there are different subsets of EMT-TFs that, though all able to induce the mesenchymal phenotype, decrease proliferation, and promote invasion, can either provide or repress CSC properties (classical EMT-TFs versus Prrx1, respectively); in other words, these EMT-TFs can either promote or repress TICs. The bottom line is that (i) EMT-TFs need to be down-regulated for metastasis to form, which

implies reversion to the epithelial phenotype and increased proliferation (Fig. 5), and (ii) the regulation of stem cell properties is independent from that of epithelial plasticity. Malignancy by cancer cells requires the reversion to the epithelial phenotype and the maintenance of a “stemness” state (70), which may be achieved when the Prrx1 type of EMT-TFs is repressed (16) and/or when connective tissue growth factor (CTGF), recently described as both a MET inducer and a stemlike properties enhancer, is activated in cancer cells (71) (Fig. 5).

EMT and MET in Cancer: Conflicting Data or Just Plasticity?

The high degree of epithelial plasticity observed during cancer progression, together with the independent regulation of EMT and stemness, has several implications. EMT-related invasion plus dissemination is required but is not sufficient for completing the metastatic cascade, and therefore invasive and metastatic may not be equivalent terms.

In agreement with the requirement for invasion and dissemination for metastasis formation, the presence of CTCs has prognostic and predictive value in breast cancer patients (72). Accordingly, abrogating EMT-TFs, such as Snail factors, in cancer cells makes these cells less prone to invade and later generate metastasis when injected in mice (18). However, constitutive expression of Snail, pRRX1, or Twist also suppresses metastasis formation (60, 67), as EMT-TFs must be down-regulated for metastasis to form (16, 60). Rather

than representing a paradox, these data simply reflect the plasticity of epithelia and the dynamics of the whole metastatic process, resembling that of embryonic cell migration to populate distant territories. Thus, whereas the EMT is associated with the early steps in metastasis (such as delamination, invasion, and intravasation), it is not associated with metastatic colonization. There is no association between the expression of Snail or Twist in primary tumors and relapse-free survival in patients with breast or lung squamous carcinomas (16). These findings are important when discussing the role of different elements in tumor progression, and the phrase “invasive and metastatic” should be avoided when referring to factors involved in the acquisition of invasive properties by cancer cells. A similar situation applies when we contemplate embryonic development. The EMT-TFs are expressed in the embryo wherever EMT processes take place. However, EMT-TFs are not expressed in the derivatives of these migratory populations, because once embryonic cells reach their destination to differentiate into distinct tissues, the EMT inducers are repressed. Thus, EMT-TFs are related to cell behavior rather than to cell fate, and hence, their expression is only transient. Accordingly, EMT-TFs cannot be used as markers of differentiated cell populations, the equivalent of differentiated distant metastases.

The description of signals that down-regulate EMT-TFs at the metastatic sites awaits further investigation, but it is clear that interactions between CSCs and the tumor microenvironment dictate (i) EMT and invasion of the primary tumor (73, 74) and (ii) colonization at the metastatic niche (57, 58, 74). The EMT program also seems to help recently extravasated cells to extend filopodium-like protrusions and interact productively with the niche, promoting homing and proliferation (75). Cytokines are important to modify both microenvironments, as they are fundamental to recruit mesenchymal stem cells and inflammatory cells. The stroma influences metastasis formation, and recent data point to the regulation of TGF β /BMP signaling in both the primary tumor and the metastatic niche in colorectal and breast carcinomas (33, 76, 77).

Toward Improved Metastatic Therapeutic Strategies

Cell plasticity and heterogeneity in tumor biology also has an impact on the design of therapeutic strategies (69). Classical cytotoxic chemotherapy has proven beneficial in patients with carcinoma. However, one of the main caveats is the appearance of tumor recurrence, associated with acquired multidrug resistance and concomitant with the appearance of signs of EMT and stemness in the residual or relapsed tumor after chemotherapy. Resistance to cell death is a property of embryonic migratory cells upon undergoing EMT, implemented to promote their arrival to their destination (78). A similar strategy is used by cancer stem cells, and therefore, efforts are being devoted to

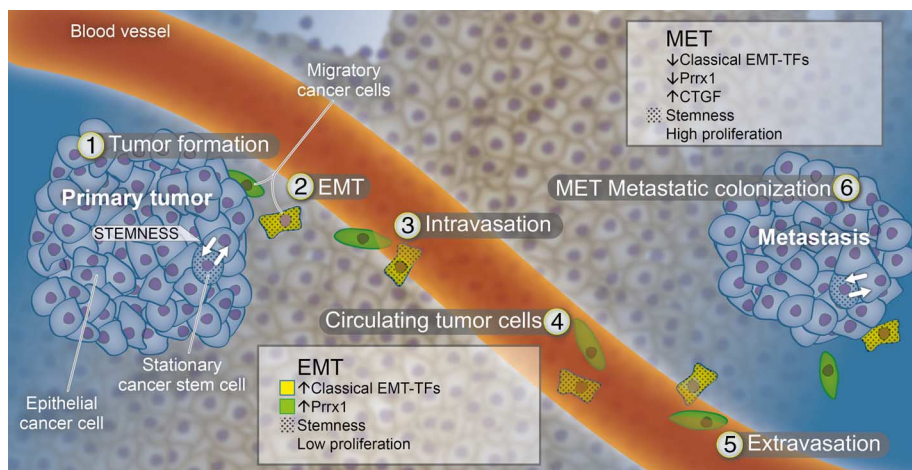


Fig. 5. Reversibility of EMT during metastatic colonization. Cancer cells delaminate from the primary tumor expressing EMT-TFs, which endow them with invasive properties to migrate through the extracellular matrix and enter the lymphatic and blood vessels. Those migratory cancer cells expressing classical EMT-TFs (Snail, Twist, and Zeb factors) present CSC properties, whereas those expressing Prrx1 do not. These two classes of CTCs are depicted, although whether the two types of EMT occur in parallel or are exclusive for individual tumors has not been shown. After extravasation, CTCs can colonize distant organs, undergoing a MET upon down-regulation of the EMT-TFs, which reverts them to the epithelial state and increases proliferation. Further work is necessary to characterize the signals that down-regulate the classical EMT-TFs while maintaining CSC properties. CTGF has been shown to induce MET concomitantly with CSC traits in cancer cells, a global effect that promotes metastatic colonization and is similar to that produced after Prrx1 down-regulation. Stationary cancer stem cells can be converted into differentiated tumor cells, and dedifferentiation of non-CSCs can generate cells with TIC. This bidirectional conversion (represented by white double arrows) adds a high degree of phenotypic plasticity in tumors that requires the design of improved therapeutic strategies.

fighting not only proliferation but also EMT and stem cell–like properties to prevent the metastatic disease in cancer patients.

To target the EMT, the strategy has been to inhibit some of its inducing signaling pathways, including those of EGF receptor, platelet-derived growth factor receptor, and TGF β . Recent data indicate that inhibiting the TGF β pathway might be beneficial in colorectal cancer metastatic disease (77). But even if the bulk of the tumor can be considerably decreased, chemoresistant stem cells will support the subsequent growth of the tumor. Therefore, different strategies have been used to directly target CSCs (including inhibitors of additional pathways such as those triggered by the developmental factors Notch, Wnt, and Shh) using cytoprotective agents and/or small molecules identified in high-throughput screenings.

Recent data indicate that stem cells can generate differentiated cells and also that adult normal and (nonstem) cancer cells can reenter the stem state, pointing to a bidirectional conversion between stem and nonstem populations (79, 80) (Fig. 5). This additional plasticity seems to be dependent on the chromatin state of the *Zeb1* promoter, which is in a bivalent state in non-CSCs so that it can be rapidly activated in response to EMT-inducing signals to help the conversion of non-CSCs into CSCs (81) (Fig. 4). Thus, therapeutic strategies should be adjusted to this superimposed level of cellular plasticity, as combating CSCs is unlikely to be sufficient because new stem cells can be generated from the remaining differentiated tumor cells. The idea is to combine classical chemotherapy with anti-CSC drugs to simultaneously hit nonstem cells and CSC in a tumor. Given the importance of the tumor microenvironment in cancer progression, therapeutic strategies should also consider targeting the stroma.

Finally, it is clear that fully preventing delamination from the primary tumor will impede metastasis, which is the principle that has inspired efforts by academia and pharmaceutical companies to block the EMT. A recent report shows that this seems to be a valid strategy, as observed in a mouse model of ovarian carcinoma (28). Nonetheless, in the light of recent data on epithelial plasticity during metastatic colonization and the need for cancer cells to revert to the epithelial phenotype through a MET (16, 60), rather than preventing metastasis, inhibiting EMT could favor the formation of secondary tumors from already disseminated cells. This is particularly important in carcinomas in which EMT is a very early event in tumorigenesis, where cancer cells have already disseminated before the tumor is diagnosed, as in pancreatic and breast carcinomas (82, 83). By contrast, as EMT reversion does not happen during the development of the fibrotic disease, therapeutic intervention inhibiting EMT is a promising strategy to ameliorate fibrosis and organ degeneration (37).

Recent advances reveal an unanticipated degree of cell plasticity during the progression of

carcinomas to the metastatic state and in the generation of CSCs, reflecting the complex biology of cancer. This concept will undoubtedly influence the strategies for therapeutic intervention. Further complexity may arise when considering other tumor types. An example is found in melanomas, where recent data show that ZEB1 and TWIST1 promote dedifferentiation and are associated with late-stage melanoma and poor prognosis. By contrast, SNAIL2 and ZEB2 do not behave as classical EMT inducers, as they are expressed in normal melanocytes and work as tumor suppressors (84). Fortunately, the process of morphogenesis provides clues as to how cell plasticity may occur in cancer, including the responses elicited by the different EMT-TFs and the mechanisms used in the maintenance and differentiation of CSCs. Together with embryos, cancer animal models such as “avatar” mice (mice that carry cancer cells from individual human patients) will help define the best therapeutic strategy, even in a personalized manner. In general terms, although inhibiting EMT may be counterproductive, strategies aimed at targeting cancer stem cells are very promising, keeping in mind that new cancer stem cells can be produced from differentiated nonstem bulk tumor cells.

References and Notes

1. H. Acloque, M. S. Adams, K. Fishwick, M. Bronner-Fraser, M. A. Nieto, Epithelial-mesenchymal transitions: The importance of changing cell state in development and disease. *J. Clin. Invest.* **119**, 1438–1449 (2009). doi: [10.1172/JCI38019](#); pmid: [19487820](#)
2. J. P. Thiery, H. Acloque, R. Y. Huang, M. A. Nieto, Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871–890 (2009). doi: [10.1016/j.cell.2009.11.007](#); pmid: [19945376](#)
3. M. A. Nieto, The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu. Rev. Cell Dev. Biol.* **27**, 347–376 (2011). doi: [10.1146/annurev-cellbio-092910-154036](#); pmid: [21740232](#)
4. H. Peinado, D. Olmeda, A. Cano, Snail, Zeb and bHLH factors in tumour progression: An alliance against the epithelial phenotype? *Nat. Rev. Cancer* **7**, 415–428 (2007). doi: [10.1038/nrc2131](#); pmid: [17508028](#)
5. C. L. Chaffer, R. A. Weinberg, A perspective on cancer cell metastasis. *Science* **331**, 1559–1564 (2011). doi: [10.1126/science.1203543](#); pmid: [21436443](#)
6. P. Navarro, E. Lozano, A. Cano, Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells. Possible involvement of plakoglobin. *J. Cell Sci.* **105**, 923–934 (1993). pmid: [8227214](#)
7. B. Ell, Y. Kang, Transcriptional control of cancer metastasis. *Trends Cell Biol.* (2013). doi: [10.1016/j.tcb.2013.06.001](#); pmid: [23838335](#)
8. H. Ledford, Cancer theory faces doubts. *Nature* **472**, 273 (2011). doi: [10.1038/472273a](#); pmid: [21512545](#)
9. D. Entenberg *et al.*, Imaging tumor cell movement in vivo. *Curr. Protoc. Cell Biol.* **58**, 19.7.1–19.7.19 (2013). pmid: [23456602](#)
10. C. Raimondi *et al.*, Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Res. Treat.* **130**, 449–455 (2011). doi: [10.1007/s10549-011-1373-x](#); pmid: [21298334](#)
11. M. Yu *et al.*, Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* **339**, 580–584 (2013). doi: [10.1126/science.1228522](#); pmid: [23372014](#)
12. Y. Kang, K. Pantel, Tumor cell dissemination: Emerging biological insights from animal models and cancer patients. *Cancer Cell* **23**, 573–581 (2013). doi: [10.1016/j.ccr.2013.04.017](#); pmid: [23680145](#)
13. T. Brabletz, EMT and MET in metastasis: Where are the cancer stem cells? *Cancer Cell* **22**, 699–701 (2012). doi: [10.1016/j.ccr.2012.11.009](#); pmid: [23238008](#)
14. R. Kalluri, R. A. Weinberg, The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119**, 1420–1428 (2009). doi: [10.1172/JCI39104](#); pmid: [19487818](#)
15. J. M. López-Novoa, M. A. Nieto, Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. *EMBO Mol. Med.* **1**, 303–314 (2009). doi: [10.1002/emmm.200900043](#); pmid: [20049734](#)
16. O. H. Ocaña *et al.*, Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prx1. *Cancer Cell* **22**, 709–724 (2012). doi: [10.1016/j.ccr.2012.10.012](#); pmid: [23201163](#)
17. M. A. Nieto, A. Cano, The epithelial-mesenchymal transition under control: Global programs to regulate epithelial plasticity. *Semin. Cancer Biol.* **22**, 361–368 (2012). doi: [10.1016/j.semcancer.2012.05.003](#); pmid: [22613485](#)
18. D. Olmeda *et al.*, Snail and Snai2 collaborate on tumor growth and metastasis properties of mouse skin carcinoma cell lines. *Oncogene* **27**, 4690–4701 (2008). doi: [10.1038/onc.2008.118](#); pmid: [18408755](#)
19. D. D. Tran, C. A. Corsa, H. Biswas, R. L. Aft, G. D. Longmore, Temporal and spatial cooperation of Snail1 and Twist1 during epithelial-mesenchymal transition predicts for human breast cancer recurrence. *Mol. Cancer Res.* **9**, 1644–1657 (2011). doi: [10.1158/1541-7786.MCR-11-0371](#); pmid: [22006115](#)
20. N. Dave *et al.*, Functional cooperation between Snail1 and twist in the regulation of ZEB1 expression during epithelial to mesenchymal transition. *J. Biol. Chem.* **286**, 12024–12032 (2011). doi: [10.1074/jbc.M110.168625](#); pmid: [21317430](#)
21. C. Scheel *et al.*, Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. *Cell* **145**, 926–940 (2011). doi: [10.1016/j.cell.2011.04.029](#); pmid: [21663795](#)
22. C.-Y. Wu, Y.-P. Tsai, M.-Z. Wu, S.-C. Teng, K.-J. Wu, Epigenetic reprogramming and post-transcriptional regulation during the epithelial-mesenchymal transition. *Trends Genet.* **28**, 454–463 (2012). doi: [10.1016/j.tig.2012.05.005](#); pmid: [22717049](#)
23. C. C. Warzecha, R. P. Carstens, Complex changes in alternative pre-mRNA splicing play a central role in the epithelial-to-mesenchymal transition (EMT). *Semin. Cancer Biol.* **22**, 417–427 (2012). doi: [10.1016/j.semcancer.2012.04.003](#); pmid: [22548723](#)
24. B. De Craene, G. Bex, Regulatory networks defining EMT during cancer initiation and progression. *Nat. Rev. Cancer* **13**, 97–110 (2013). doi: [10.1038/nrc3447](#); pmid: [23344542](#)
25. C. C. Warzecha *et al.*, An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *EMBO J.* **29**, 3286–3300 (2010). doi: [10.1038/emboj.2010.195](#); pmid: [20711167](#)
26. O. G. McDonald, H. Wu, W. Timp, A. Doi, A. P. Feinberg, Genome-scale epigenetic reprogramming during epithelial-to-mesenchymal transition. *Nat. Struct. Mol. Biol.* **18**, 867–874 (2011). doi: [10.1038/nsmb.2084](#); pmid: [21725293](#)
27. A. Rada-Iglesias *et al.*, Epigenomic annotation of enhancers predicts transcriptional regulators of human neural crest. *Cell Stem Cell* **11**, 633–648 (2012). doi: [10.1016/j.stem.2012.07.006](#); pmid: [22981823](#)
28. D. Yang *et al.*, Integrated analyses identify a master microRNA regulatory network for the mesenchymal subtype in serous ovarian cancer. *Cancer Cell* **23**, 186–199 (2013). doi: [10.1016/j.ccr.2012.12.020](#); pmid: [23410973](#)
29. E. Theveneau *et al.*, Collective chemotaxis requires contact-dependent cell polarity. *Dev. Cell* **19**, 39–53 (2010). doi: [10.1016/j.devcel.2010.06.012](#); pmid: [20643349](#)
30. D. Sarrió *et al.*, Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res.* **68**, 989–997 (2008). doi: [10.1158/0008-5472.CAN-07-2017](#); pmid: [18281472](#)
31. M. Zeisberg *et al.*, Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition.

- J. Biol. Chem. **282**, 23337–23347 (2007). doi: [10.1074/jbc.M700194200](#); pmid: [17562716](#)
32. P. Rørth, Collective cell migration. *Annu. Rev. Cell Dev. Biol.* **25**, 407–429 (2009). doi: [10.1146/annurev.cellbio.042308.113231](#); pmid: [19575657](#)
33. S. Giampieri *et al.*, Localized and reversible TGF β signalling switches breast cancer cells from cohesive to single cell motility. *Nat. Cell Biol.* **11**, 1287–1296 (2009). doi: [10.1038/ncb1973](#); pmid: [19838175](#)
34. E. D. Hay, Collagen and other matrix proteins in embryogenesis, in *Cell Biology of the Extracellular Matrix*, E. D. Hay, Ed. (Plenum Press, New York, 1991).
35. A. T. Dudley, K. M. Lyons, E. J. Robertson, A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795–2807 (1995). doi: [10.1101/gad.9.22.2795](#); pmid: [7590254](#)
36. P. Samavarchi-Tehrani *et al.*, Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* **7**, 64–77 (2010). doi: [10.1016/j.stem.2010.04.015](#); pmid: [20621051](#)
37. M. Zeisberg, A. A. Shah, R. Kalluri, Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. *J. Biol. Chem.* **280**, 8094–8100 (2005). doi: [10.1074/jbc.M413102200](#); pmid: [15591043](#)
38. J. T. Buijs *et al.*, BMP7, a putative regulator of epithelial homeostasis in the human prostate, is a potent inhibitor of prostate cancer bone metastasis in vivo. *Am. J. Pathol.* **171**, 1047–1057 (2007). doi: [10.2353/ajpath.2007.070168](#); pmid: [17724140](#)
39. T. Watanabe, Y. Sato, D. Saito, R. Tadokoro, Y. Takahashi, EphrinB2 coordinates the formation of a morphological boundary and cell epithelialization during somite segmentation. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 7467–7472 (2009). doi: [10.1073/pnas.0902859106](#); pmid: [19380726](#)
40. T. J. Carroll, J. S. Park, S. Hayashi, A. Majumdar, A. P. McMahon, Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev. Cell* **9**, 283–292 (2005). doi: [10.1016/j.devcel.2005.05.016](#); pmid: [16054034](#)
41. A. Boutet *et al.*, Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney. *EMBO J.* **25**, 5603–5613 (2006). doi: [10.1038/sj.emboj.7601421](#); pmid: [17093497](#)
42. J. K. Dale *et al.*, Oscillations of the snail genes in the presomitic mesoderm coordinate segmental patterning and morphogenesis in vertebrate somitogenesis. *Dev. Cell* **10**, 355–366 (2006). doi: [10.1016/j.devcel.2006.02.011](#); pmid: [16516838](#)
43. T. Brabletz *et al.*, Variable β -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10356–10361 (2001). doi: [10.1073/pnas.171610498](#); pmid: [11526241](#)
44. K. Gumireddy *et al.*, KLF17 is a negative regulator of epithelial-mesenchymal transition and metastasis in breast cancer. *Nat. Cell Biol.* **11**, 1297–1304 (2009). doi: [10.1038/ncb1974](#); pmid: [19801974](#)
45. R. Chakrabarti *et al.*, E1f5 inhibits the epithelial-mesenchymal transition in mammary gland development and breast cancer metastasis by transcriptionally repressing Snail2. *Nat. Cell Biol.* **14**, 1212–1222 (2012). doi: [10.1038/ncb2607](#); pmid: [23086238](#)
46. K. C. Scribner, F. Behbod, W. W. Porter, Regulation of DCIS to invasive breast cancer progression by Single-minded-2s (SIM2s). *Oncogene* **32**, 2631–2639 (2013). doi: [10.1038/ncb2620](#); pmid: [22777354](#)
47. Y. Kasai, J. R. Nambu, P. M. Lieberman, S. T. Crews, Dorsal-ventral patterning in Drosophila: DNA binding of snail protein to the single-minded gene. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3414–3418 (1992). doi: [10.1073/pnas.89.8.3414](#); pmid: [1533042](#)
48. S. Brabletz, T. Brabletz, The ZEB/miR-200 feedback loop—A motor of cellular plasticity in development and cancer? *EMBO Rep.* **11**, 670–677 (2010). doi: [10.1038/embor.2010.117](#); pmid: [20706219](#)
49. Y. Yang *et al.*, The Notch ligand Jagged2 promotes lung adenocarcinoma metastasis through a miR-200-dependent pathway in mice. *J. Clin. Invest.* **121**, 1373–1385 (2011). doi: [10.1172/JCI42579](#); pmid: [21403400](#)
50. H. Siemens *et al.*, miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. *Cell Cycle* **10**, 4256–4271 (2011). doi: [10.4161/cc.10.24.18552](#); pmid: [22134354](#)
51. S. J. Song *et al.*, MicroRNA-antagonism regulates breast cancer stemness and metastasis via TET-family-dependent chromatin remodeling. *Cell* **154**, 311–324 (2013). doi: [10.1016/j.cell.2013.06.026](#); pmid: [23830207](#)
52. T. Kim *et al.*, p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J. Exp. Med.* **208**, 875–883 (2011). doi: [10.1084/jem.20110235](#); pmid: [21518799](#)
53. P. Dong *et al.*, Mutant p53 gain-of-function induces epithelial-mesenchymal transition through modulation of the miR-130b-ZEB1 axis. *Oncogene* **32**, 3286–3295 (2013). doi: [10.1038/ncb2012.334](#); pmid: [22847613](#)
54. M. Steder *et al.*, DNP73 exerts function in metastasis initiation by disconnecting the inhibitory role of EPLIN on IGF1R-AKT/STAT3 signaling. *Cancer Cell* **24**, 512–527 (2013). doi: [10.1016/j.ccr.2013.08.023](#)
55. T. Spruce *et al.*, An early developmental role for miRNAs in the maintenance of extraembryonic stem cells in the mouse embryo. *Dev. Cell* **19**, 207–219 (2010). doi: [10.1016/j.devcel.2010.07.014](#); pmid: [20708584](#)
56. C. Peng *et al.*, A unilateral negative feedback loop between miR-200 microRNAs and Sox2/E2F3 controls neural progenitor cell-cycle exit and differentiation. *J. Neurosci.* **32**, 13292–13308 (2012). doi: [10.1523/JNEUROSCI.2124-12.2012](#); pmid: [22993445](#)
57. M. Korpai *et al.*, Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat. Med.* **17**, 1101–1108 (2011). doi: [10.1038/nm.2401](#); pmid: [21822286](#)
58. C. L. Chaffer *et al.*, Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: Role of fibroblast growth factor receptor-2. *Cancer Res.* **66**, 11271–11278 (2006). doi: [10.1158/0008-5472.CAN-06-2044](#); pmid: [17145872](#)
59. D. Gao *et al.*, Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. *Cancer Res.* **72**, 1384–1394 (2012). doi: [10.1158/0008-5472.CAN-11-2905](#); pmid: [22282653](#)
60. J. H. Tsai, J. L. Donaher, D. A. Murphy, S. Chau, J. Yang, Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell* **22**, 725–736 (2012). doi: [10.1016/j.ccr.2012.09.022](#); pmid: [23201165](#)
61. S. A. Mani *et al.*, The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704–715 (2008). doi: [10.1016/j.cell.2008.03.027](#); pmid: [18485877](#)
62. A. P. Morel *et al.*, Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLOS ONE* **3**, e2888 (2008). doi: [10.1371/journal.pone.0002888](#); pmid: [18682804](#)
63. T. Brabletz, A. Jung, S. Spaderna, F. Hlubek, T. Kirchner, Opinion: Migrating cancer stem cells—An integrated concept of malignant tumor progression. *Nat. Rev. Cancer* **5**, 744–749 (2005). doi: [10.1038/nrc1694](#); pmid: [16148886](#)
64. R. Li *et al.*, A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* **7**, 51–63 (2010). doi: [10.1016/j.stem.2010.04.014](#); pmid: [20621050](#)
65. K. Polyak, R. A. Weinberg, Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits. *Nat. Rev. Cancer* **9**, 265–273 (2009). doi: [10.1038/nrc2620](#); pmid: [19262571](#)
66. J. M. Polo *et al.*, A molecular roadmap of reprogramming somatic cells into iPS cells. *Cell* **151**, 1617–1632 (2012). doi: [10.1016/j.cell.2012.11.039](#); pmid: [23260147](#)
67. T. Celià-Terrassa *et al.*, Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. *J. Clin. Invest.* **122**, 1849–1868 (2012). doi: [10.1172/JCI59218](#); pmid: [22505459](#)
68. W. Guo *et al.*, Slug and Sox9 cooperatively determine the mammary stem cell state. *Cell* **148**, 1015–1028 (2012). doi: [10.1016/j.cell.2012.02.008](#); pmid: [22385965](#)
69. N. D. Marjanovic, R. A. Weinberg, C. L. Chaffer, Cell plasticity and heterogeneity in cancer. *Clin. Chem.* **59**, 168–179 (2013). doi: [10.1373/clinchem.2012.184655](#); pmid: [23202226](#)
70. B. J. Van Denderen, E. W. Thompson, Cancer: The to and fro of tumour spread. *Nature* **493**, 487–488 (2013). doi: [10.1038/493487a](#); pmid: [23344357](#)
71. C. C. Chang *et al.*, Connective tissue growth factor activates pluripotency genes and mesenchymal-epithelial transition in head and neck cancer cells. *Cancer Res.* **73**, 4147–4157 (2013). doi: [10.1158/0008-5472.CAN-12-4085](#); pmid: [23687336](#)
72. J. Y. Pierga *et al.*, High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. *Ann. Oncol.* **23**, 618–624 (2012). doi: [10.1093/annonc/mdr263](#); pmid: [21642515](#)
73. A. E. Karnoub *et al.*, Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**, 557–563 (2007). doi: [10.1038/nature06188](#); pmid: [17914389](#)
74. I. Malanchi *et al.*, Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature* **481**, 85–89 (2012). doi: [10.1038/nature10694](#); pmid: [22158103](#)
75. T. Shibue, M. W. Brooks, R. A. Weinberg, An integrin-linked machinery of cytoskeletal regulation that enables experimental tumor initiation and metastatic colonization. *Cancer Cell* **24**, 481–498 (2013). doi: [10.1016/j.ccr.2013.08.012](#)
76. H. Gao *et al.*, The BMP inhibitor Coco reactivates breast cancer cells at lung metastatic sites. *Cell* **150**, 764–779 (2012). doi: [10.1016/j.cell.2012.06.035](#); pmid: [22901808](#)
77. A. Calon *et al.*, Dependency of colorectal cancer on a TGF- β -driven program in stromal cells for metastasis initiation. *Cancer Cell* **22**, 571–584 (2012). doi: [10.1016/j.ccr.2012.08.013](#); pmid: [23153532](#)
78. S. Vega *et al.*, Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev.* **18**, 1131–1143 (2004). doi: [10.1101/gad.294104](#); pmid: [15155580](#)
79. C. L. Chaffer *et al.*, Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 7950–7955 (2011). doi: [10.1073/pnas.1102454108](#); pmid: [21498687](#)
80. S. Schwitala *et al.*, Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* **152**, 25–38 (2013). doi: [10.1016/j.cell.2012.12.012](#); pmid: [23273993](#)
81. C. L. Chaffer *et al.*, Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. *Cell* **154**, 61–74 (2013). doi: [10.1016/j.cell.2013.06.005](#); pmid: [23827675](#)
82. Y. Hüsemann *et al.*, Systemic spread is an early step in breast cancer. *Cancer Cell* **13**, 58–68 (2008). doi: [10.1016/j.ccr.2007.12.003](#); pmid: [18167340](#)
83. A. D. Rhim *et al.*, EMT and dissemination precede pancreatic tumor formation. *Cell* **148**, 349–361 (2012). doi: [10.1016/j.cell.2011.11.025](#); pmid: [22265420](#)
84. J. Caramel *et al.*, A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. *Cancer Cell* **24**, 466–480 (2013). doi: [10.1016/j.ccr.2013.08.018](#); pmid: [24075834](#)

Acknowledgments: I thank members of my lab for helpful discussions, in particular R. Corcoles for contributing the Review Summary figure and movies S1 and S2. I would also like to thank S. Ingham (Instituto de Neurociencias de Alicante—CSIC—UMH) for his help with the figures. Work in my lab is supported by grants from the Spanish Ministry of Science and Innovation (BFU2008-01042 and CONSOLIDER-INGENIO 2010 CSD2007-00017 and 00023), the Generalitat Valenciana (Prometeo II/2013/002 and ISIC 2012/010), and the European Research Council (ERC AdG322694).

Supplementary Materials
www.sciencemag.org/content/342/6159/1234850/suppl/DC1
 Movies S1 and S2

10.1126/science.1234850

Architecture of an RNA Polymerase II Transcription Pre-Initiation Complex

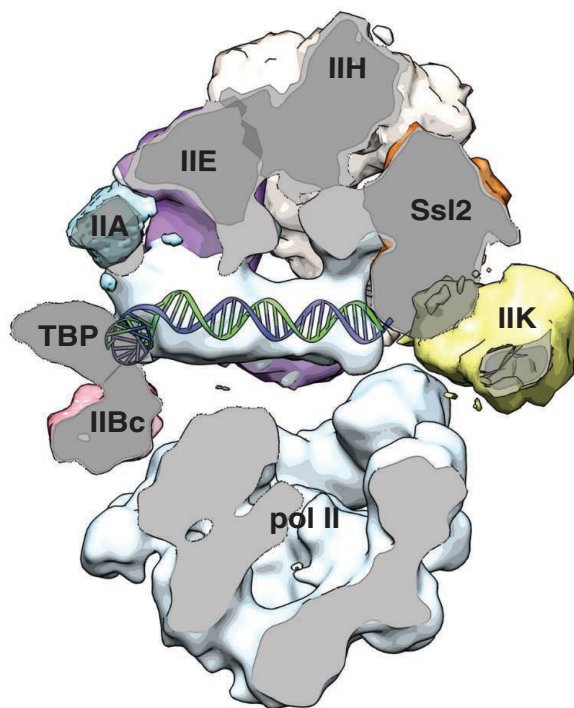
Kenji Murakami, Hans Elmlund, Nir Kalisman, David A. Bushnell, Christopher M. Adams, Maia Azubel, Dominika Elmlund, Yael Levi-Kalisman, Xin Liu, Brian J. Gibbons, Michael Levitt, Roger D. Kornberg*

Introduction: RNA polymerase II (pol II) is capable of RNA synthesis but is unable to recognize a promoter or to initiate transcription. For these essential functions, a set of general transcription factors (GTFs)—termed TFIIB, -D, -E, -F, and -H—is required. The GTFs escort promoter DNA through the stages of recruitment to pol II, unwinding to create a transcription bubble, descent into the pol II cleft, and RNA synthesis to a length of 25 residues and transition to a stable elongating complex. The structural basis for these transactions is largely unknown. Only TFIIB has been solved by means of x-ray diffraction, in a complex with pol II. We report on the structure of a complete set of GTFs, assembled with pol II and promoter DNA in a 32-protein, 1.5 megaDalton “pre-initiation complex” (PIC), as revealed with cryo-electron microscopy (cryo-EM) and chemical cross-linking.

Methods: Three technical advances enabled the structural analysis of the PIC. First, a procedure was established for the preparation of a stable, abundant PIC. Both the homogeneity and functional activity of the purified PIC were demonstrated. Second, an algorithm was developed for alignment of cryo-EM images that requires no prior information (no “search model”) and that can distinguish multiple conformational states. Last, a computational method was devised for determining the arrangement of protein subunits and domains within a cryo-EM density map from a pattern of chemical cross-linking.

Results: The density map of the PIC showed a pronounced division in two parts, one pol II and the other the GTFs. Promoter DNA followed a straight path, in contact with the GTFs but well separated from pol II, suspended above the active center cleft. Cross-linking and computational analysis led to a most probable arrangement of the GTFs, with IIB at the upstream end of the pol II cleft, followed by IIF, IIE, and IIH. The Ssl2 helicase subunit of IIH was located at the downstream end of the cleft.

Discussion: A principle of the PIC revealed by this work is the interaction of promoter DNA with the GTFs and not with pol II. The GTFs position the DNA above the pol II cleft, but interaction with pol II can only occur after melting of the DNA to enable bending for entry in the cleft. Contact of the DNA with the Ssl2 helicase in the PIC leads to melting (in the presence of adenosine triphosphatase). Cryo-EM by others, based on sequential assembly and analysis of partial complexes rather than of the complete PIC, did not show a separation between pol II and GTFs and revealed direct DNA–pol II interaction. The discrepancy calls attention to a role of the GTFs in preventing direct DNA–polymerase interaction.



READ THE FULL ARTICLE ONLINE
<http://dx.doi.org/10.1126/science.1238724>



Cite this article as K. Murakami *et al.*,
Science 342, 1238724 (2013).
 DOI: 10.1126/science.1238724

FIGURES IN THE FULL ARTICLE

Fig. 1. Cryo-EM structure of the 32-protein PIC including TFIIS (PIC).

Fig. 2. Location of TFIH in the PIC.

Fig. 3. Locations of general transcription factors and promoter DNA in the PIC.

Fig. 4. Spatial restraints from XL-MS: domains of TFIIF.

Fig. 5. Combination of XL-MS and cryo-EM: TFIIE and TFIH.

Fig. 6. Comparison of reconstructed volume of the PIC in this work to that of negatively stained human PIC.

Fig. 7. Comparison of DNA paths within PIC structures.

SUPPLEMENTARY MATERIALS

Materials and Methods
 Supplementary Text
 Figs. S1 to S14
 Tables S4 and S5
 References (46–63)
 Tables S1 to S3
 Movies S1 to S3
 Protein Data Bank file for the PIC model

A section through the cryo-EM structure of the complete PIC. Cut surfaces are shown in gray. Locations of densities due to pol II and the GTFs (TFIIA, TFIIB C-terminal domain, TBP subunit of TFIID, TFIIE, and TFIH, including its helicase subunit Ssl2 and its kinase module TFIK) are indicated. Density due to DNA is indicated by the superimposed double helix model. TFIIF is not seen in this section.

The list of author affiliations is available in the full article online.

*Corresponding author. E-mail: kornberg@stanford.edu

Architecture of an RNA Polymerase II Transcription Pre-Initiation Complex

Kenji Murakami,^{1*} Hans Elmlund,^{1*} Nir Kalisman,^{1*} David A. Bushnell,¹ Christopher M. Adams,² Maia Azubel,¹ Dominika Elmlund,¹ Yael Levi-Kalisman,^{1†} Xin Liu,^{1‡} Brian J. Gibbons,¹ Michael Levitt,¹ Roger D. Kornberg^{1§}

The protein density and arrangement of subunits of a complete, 32-protein, RNA polymerase II (pol II) transcription pre-initiation complex (PIC) were determined by means of cryogenic electron microscopy and a combination of chemical cross-linking and mass spectrometry. The PIC showed a marked division in two parts, one containing all the general transcription factors (GTFs) and the other pol II. Promoter DNA was associated only with the GTFs, suspended above the pol II cleft and not in contact with pol II. This structural principle of the PIC underlies its conversion to a transcriptionally active state; the PIC is poised for the formation of a transcription bubble and descent of the DNA into the pol II cleft.

Sixty proteins assemble in a 3-million Dalton complex at every RNA polymerase II (pol II) promoter, before every round of transcription (1, 2). About half of these proteins, some 30 in number [the subunits of pol II and the general transcription factors (GTFs)], form a pre-initiation complex (PIC) that can recognize a minimal (TATA-box) promoter, select a transcription start site, and synthesize a nascent transcript. The remaining proteins are needed for recognition of an extended promoter (TAF complex) and for the regulation of transcription (Mediator complex) (3). Orchestration of the initiation process depends on the organization of components of the PIC. We report here on the three-dimensional (3D) arrangement of the 32 proteins of the PIC.

Biochemical studies have identified functions of several PIC proteins (1). TATA box-binding protein (TBP), a subunit of the general transcription factor TFIID, binds and bends TATA-box DNA (4). TFIIB brings the TBP-promoter DNA complex to pol II near the active center cleft (5). TFIIF, an 11-protein complex, has multiple catalytic activities, including helicase, Ssl2 (6), that melts promoter DNA to form the so-called “transcription bubble,” and a protein kinase (7) whose action upon the C-terminal domain of pol II controls association with Mediator and other accessory proteins (8). Functional roles of additional GTFs TFIIA, TFIIE, and TFIIF are less well established.

Structural information on the PIC is incomplete. X-ray structures have been determined for

pol II (9, 10), pol II-DNA complexes (11), a pol II-TFIIB complex (12–14), and a TBP-TFIIB-DNA complex (15). Structures at much lower resolution have been determined by means of electron microscopy (EM) of negatively stained proteins for a pol II-TFIIF complex (16), for TFIIE (17),

and for TFIIF (18–20). The advance reported here, structural analysis of a fully assembled PIC, was made possible by three technical developments. First, improved methods of preparation of the GTFs (21) and their assembly with pol II resulted in abundant, homogeneous PIC (22). Second, upon structural analysis by means of cryogenic electron microscopy (cryo-EM), it emerged that conformational heterogeneity was an impediment to image processing of the PIC. An algorithm was developed for classifying images on the basis of conformational state, enabling averaging and 3D reconstruction (23, 24). Last, spatial proximities of proteins in the PIC were determined by means of cross-linking and mass spectrometry (XL-MS) (25–27). A computational approach was devised for combining information from cryo-EM and XL-MS to arrive at a complete 3D map of the protein components of the PIC. Abbreviated results and discussion follow; additional results, discussion, and material and methods are presented in supplementary materials.

Cryo-EM and 3-D Reconstruction

Pol II and GTFs, isolated from the yeast *Saccharomyces cerevisiae*, were combined with a fragment of *HIS4* promoter DNA (–81/+1) and sedimented

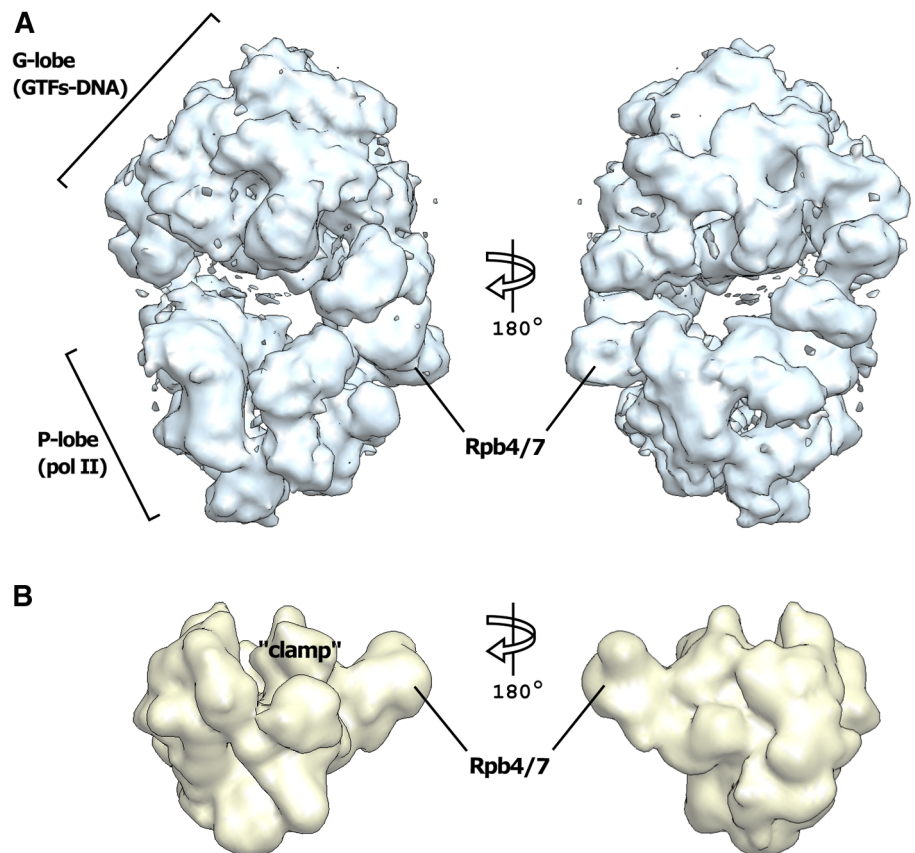


Fig. 1. Cryo-EM structure of the 32-protein PIC including TFIIS (PIC). (A) Front (left) and back (right) views of the most populated state of the PIC. (B) Low-pass filtered (20 Å) 12-subunit pol II (45) is viewed in the same orientations as in (A). The mobile “clamp” domain of pol II (10) and the dissociable subunits Rpb4/7 are indicated.

¹Department of Structural Biology, Stanford University, Stanford, CA 94305, USA. ²Stanford University Mass Spectrometry, Stanford University, Stanford, CA 94305, USA.

*These authors contributed equally to this work.

†Present address: Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, 84105 Beer-Sheva, Israel.

‡Present address: Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, Dallas, TX 75390–8511, USA.

§Corresponding author. E-mail: kornberg@stanford.edu

in a glycerol gradient as described (fig. S1) (22). Two forms of the PIC were prepared in this way: one, denoted complete PIC, contained all GTFs (TFIIA, TFIIB, TBP, TFIIE, TFIIIF, and TFIIH) and in addition the transcription elongation factor TFIIS, implicated through genetic analysis in the initiation of transcription *in vivo* (28) and with nuclear extract *in vitro* (29); a second form, denoted PIC-ΔTFIIK, was identical with the first, except for the removal of a three-subunit module of TFIIH termed TFIK (within which resides the protein kinase mentioned above). Partial complexes, lacking one or more of the GTFs, are unstable and dissociate upon handling. It is uncertain whether partial complexes adopt defined structures. Only the complete PIC (or PIC-ΔTFIIK) exhibited a barrier to digestion by exonuclease III from the downstream end of the DNA. No barrier was observed when any one of the GTFs was omitted (fig. S1). Even the complete PIC tended to dissociate during specimen preparation for cryo-EM, and glutaraldehyde was included in the glycerol gradient (30) for stabilization. Images were acquired on a transmission electron microscope equipped with a field emission gun under low-dose conditions (10 to 15 $e^-/\text{\AA}^2$) (fig. S2): 433,916 images of single particles of complete PIC and 305,160 images of PIC-ΔTFIIK.

Image processing was performed with SIMPLE (24), a program package for the analysis of asym-

metrical and heterogeneous single particles that requires no prior knowledge of the structure, no search model, and therefore does not introduce model bias. SIMPLE addresses the problem of heterogeneity by means of a “Fourier common lines” approach (23). Analysis with SIMPLE revealed two predominant states in both forms of the PIC. After refinement, the resolution of the density maps was highest for complete PIC (16 $\text{\AA}$ by the Fourier shell criterion). The maps measured $240 \times 170 \times 150$ $\text{\AA}$. In both forms of the PIC, the maps were clearly divided in two parts, confirmed through automatic segmentation, and termed P-lobe and G-lobe (Fig. 1). An excellent fit of the pol II x-ray structure (contoured at 20 $\text{\AA}$ resolution) (Fig. 1B) to the P-lobe served to validate the segmentation and the reconstructions.

Segmentation of Electron Density: Location of GTFs and DNA

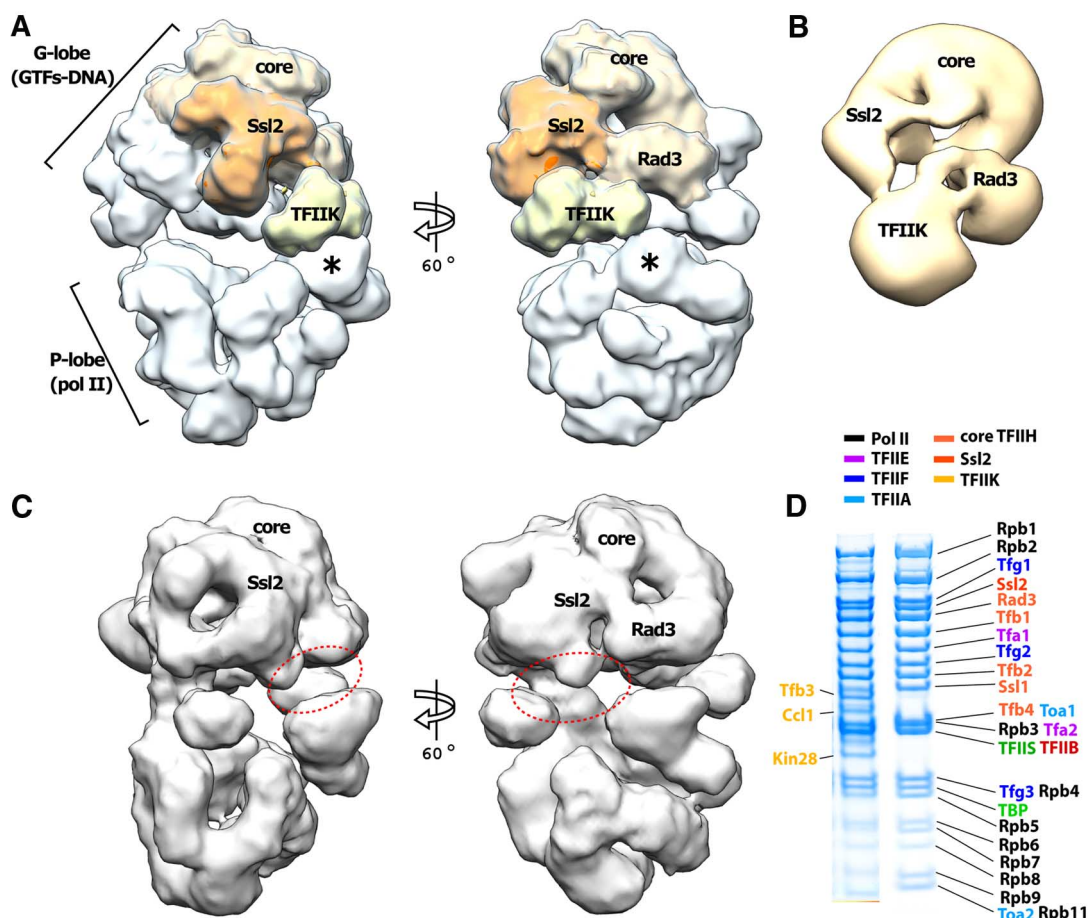
Manual fitting of the pol II structure to the P-lobe was optimized with computational refinement. A difference map calculated between the pol II structure and the P-lobe revealed one extra region of density in the P-lobe, adjacent to the Rpb4/7 subunits, which may be due to the C-terminal domain (CTD) of Rpb1 (fig. S3). The G-lobe showed similarity in size and shape to EM structures of negatively stained complete (“holo”) TFIH (Fig. 2, A and B) (20). On this basis, approximate lo-

cations of Rad3 and Ssl2 subunits of TFIH were identified (color code to all subunits of GTFs is provided in Fig. 2D), and TFIK was placed in close proximity to its substrate, the CTD. The location of TFIK was confirmed by its removal from the PIC: The density attributed to TFIK was absent from structures of PIC-ΔTFIIK (Fig. 2C).

The EM maps of the complete PIC also contained density attributable to TBP, TFIIA, TFIIB, and promoter DNA (Fig. 3). A crystallographic model (13, 14) of a “minimal” PIC (pol II-TBP-TFIIB-DNA complex) could be docked to the EM density with only slight deviations in the DNA path. The DNA density contacted G-lobe density, 10 to 20 base pairs (bp) downstream of the TATA box and merged with Ssl2 density at the downstream end (Fig. 3, A and B). Because roughly cylindrical density for DNA could be seen between the upstream and downstream points of contact, it was essentially free in this region.

Removal of density due to TBP, TFIIA, TFIIB, TFIH, and DNA from the G-lobe leaves residual density that may be attributed to TFIIE and TFIIIF. The residual density was automatically segmented into regions of ~ 100 and 90 kD, which were assigned to TFIIE and TFIIIF, respectively (Fig. 4A) on the basis of additional evidence from protein-protein cross-linking described below. Together, TFIIE and TFIIIF would largely surround the DNA in the vicinity of the TATA box.

Fig. 2. Location of TFIH in the PIC. (A) Front (left) and side (right) views of the PIC, with suggested assignments based on comparison with (B). Core and Rad3 are gold, Ssl2 is orange, and TFIK is lime yellow. The extra density marked by the asterisk may be due to the CTD of Rpb1. (B) Molecular envelope of negatively stained TFIH (20), viewed in the same orientation as (A), right. (C) Front (left) and side (right) views of the PIC lacking TFIK (PIC-ΔTFIIK). Dashed ellipses indicate the location of the density, labeled TFIK in (A), that is not observed. (D) SDS-polyacrylamide gel electrophoresis of peak glycerol gradient fractions of PIC (left) and PIC-ΔTFIIK (right). Rpb10, Rpb12, and Tfb5 are not seen because of their low molecular weights. The same color scheme is used in all figures.



XL-MS: Location of TFIIIF

The proposed locations of the GTFs within the G-lobe, based on docking and automatic segmentation, were confirmed by means of XL-MS (25–27). For this purpose, the PIC was cross-linked with BS³, a bifunctional amino group reagent. After protease digestion and mass spectrometry, assignments of cross-linked peptides to observed ion masses were scored for significance as described (27). A total of 109 intermolecular and 157 intramolecular cross-links of high significance (less than 1.5% false-positive rate) were identified (tables S1 and S2 and fig. S7). Validation came from 73 cross-links between residues separated by distances known from crystal structures of pol II-TFIIB (14), pol II-TFIIS (31), and TFIIA-TBP-DNA (32). The Ca-Ca distances

between such residues were less than 30 Å for 70 cross-links, less than 34 Å for two cross-links, and 60 Å for the remaining one. These numbers are in accord with previous studies showing that the Ca-Ca distance of residues bridged by BS³ are generally less than 30 Å, and in rare cases as much as 35 Å (25, 27). The one cross-link of 60 Å is likely a wrong assignment, which is not unexpected given the 1.5% false-positive rate.

Cross-links involving the Tfg1-Tfg2 dimerization and the Tfg2 winged-helix (WH) domains of TFIIIF were sufficient to locate these domains in the EM map (Fig. 3B). The “flexible arm” of Tfg1 and the “insertion loop” of Tfg2 (33) formed a pattern of cross-links with residues in the Rpb2 subunit of pol II (K87, K344, K358, and K426) that placed the dimerization domain adjacent to

the Rpb2 “protrusion” and “jaw-lobe” domains (Fig. 4C). The location of the insertion loop is supported by cross-links to the C-terminal region of Ssl2, which penetrates the downstream end of the pol II cleft, as shown by cross-linking of this region of Ssl2 to Rpb1 residues K1217, K1246, and K1262 (Fig. 4, A to C). The WH domain of Tfg2 also formed cross-links to the Toa2 subunit of TFIIA (K119), to TFIIB (K199) and to the Tfa2 subunit of TFIIIE (Fig. 4B), that defined a specific location along the path of the DNA (table S4). The resulting model is in excellent agreement with previous FeBABE cleavage mapping of protein-promoter DNA interaction in complexes formed in yeast nuclear extract (34).

Combination of XL-MS and Cryo-EM: Locations of TFIIIE and TFIIH

The two subunits of TFIIIE and seven subunits of core TFIIH formed many cross-links with one another (fig. S7). To interpret this information in terms of the EM map, we represented each subunit by one or two spheres (fig. S12), depending on the mass of the subunit and the pattern of internal cross-links (a total of 12 spheres) (supplementary materials). Twelve locations for these spheres, spanning the EM density attributed to TFIIIE and TFIIH (Fig. 5, A and B), were chosen by an objective procedure: The first location was at the point of highest EM density, the next location was at the point of next highest density that was more than 35 Å from the first, and so forth. There are 12 factorial (480 million) different models that assign the 12 spheres to the 12 locations, and we assessed them exhaustively. We first discarded models in which two spheres belonging to the same protein were more than 45 Å apart, reducing the number of models to half a million. We then evaluated the fit of a model to the pattern of cross-links on the basis of two measures: serious violation, defined by a pair of spheres located more than 65 Å apart in the model, for which a cross-link is nevertheless observed; and violation distance, defined as the excess over 40 Å between a pair of spheres in the model for which a cross-link is observed. These two measures were correlated over a wide range of values (Fig. 5B).

The model with the smallest sum of violation distances (Fig. 5A) was identical to the consensus of the 10 next best models at each location (Fig. 5D) and was the model returned most frequently by bootstrap analysis (fig. S13). The locations of TFIIIE and TFIIH were the same as those determined with automatic segmentation of the EM density. The size and shape of the region assigned to core TFIIH were closely similar to those of a structure determined with 2D crystallography (Fig. 5C) (18). TFIIIE extended across the surface of the G-lobe in proximity to the promoter DNA (Fig. 5A). Tfa1 and Tfa2 subunits of TFIIIE (“Tfa1 N-term” and two WH domains of Tfa2) were cross-linked to each other immediately downstream of the TATA box (between DNA -54 and -44). “Tfa1 C-term” formed additional cross-links with all subunits of core TFIIH, reaching as far as Ssl1 (the N-terminal

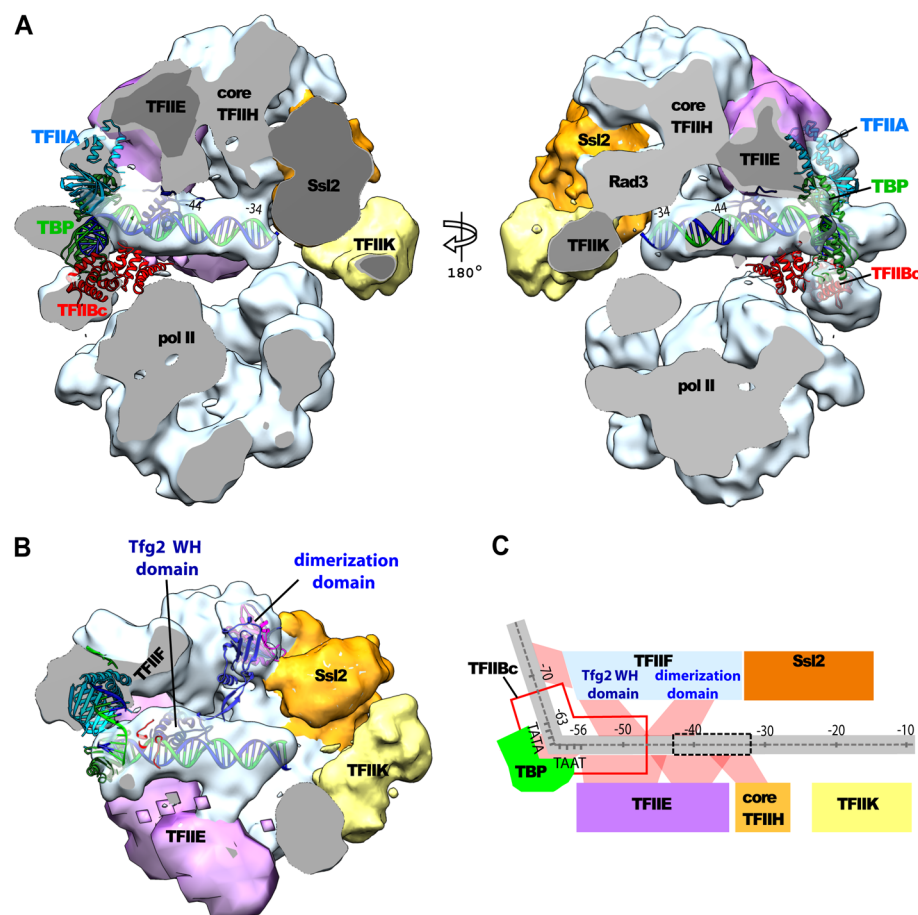


Fig. 3. Locations of general transcription factors and promoter DNA in the PIC. (A) The PIC structure, sectioned to reveal the DNA in the center (cut surfaces are gray). The structure on the left side of Fig. 1A was rotated 60° counterclockwise about the vertical axis (left) or 120° clockwise (right). Subunits of TFIIIE and TFIIH (core TFIIH and its Rad3 component, Ssl2, and TFIIC) are indicated. Atomic models of the TBP (green)—TFIIBc (red)—TATA-box complex (15) and TFIIA (blue) (32) were fitted to the cryo-EM density and displayed as ribbon diagrams. The TATA box DNA was extended with straight B-form DNA with minor adjustments of the DNA path. (B) The underside of the G-lobe, viewed from bottom of (A), left, with the P-lobe removed. Atomic models of the Tfg1-Tfg2 dimerization domain (Tfg1, blue; Tfg2, magenta) and the winged helix (WH) domain of Tfg2 (dark blue) were located by means of protein-protein cross-linking and displayed as ribbon diagrams. (C) Schematic diagram of proximity relationships of promoter DNA (gray) and GTFs in the PIC. Positions in the DNA are numbered with respect to the first transcription start site of the *HIS4* promoter. The TATA box spans positions from -63 to -56. Transcription bubble formed upon initial promoter melting is indicated by the dashed box. Putative interactions are indicated with red shading.

half, denoted "Ssl1 N"). Ssl1 N formed cross-links to all subunits of core TFIIB, which is consistent with its assignment in the 2D crystal structure (18) to a central position, between Rad3 and the rest of the structure (Fig. 5C, right).

Discussion

This study has revealed a central principle of the PIC: the association of promoter DNA only with the GTFs and not with pol II. Promoter DNA is

suspended above the pol II cleft, contacting three GTFs—TFIIB, TFIID (TBP subunit), and TFIIE—at the upstream end of the cleft (TATA box) and contacting TFIIB (Ssl2 helicase subunit) at the downstream end. In between, the DNA is free and available for action of the helicase, which untwists the DNA to introduce negative superhelical strain and thereby promote melting at a distance (35).

This principle of the PIC is a consequence of the rigidity of duplex DNA. The promoter du-

plex must follow a straight path, whereas bending through $\sim 90^\circ$ is required for binding in the pol II cleft (11). Only after melting can the DNA bend for entry in the cleft. Melting is thermally driven, induced by untwisting strain in the DNA above the cleft. A melted region is short-lived and must be captured by binding to pol II, which occurs rapidly enough because the DNA is positioned above the cleft. The GTFs therefore catalyze the formation of a stably melted region

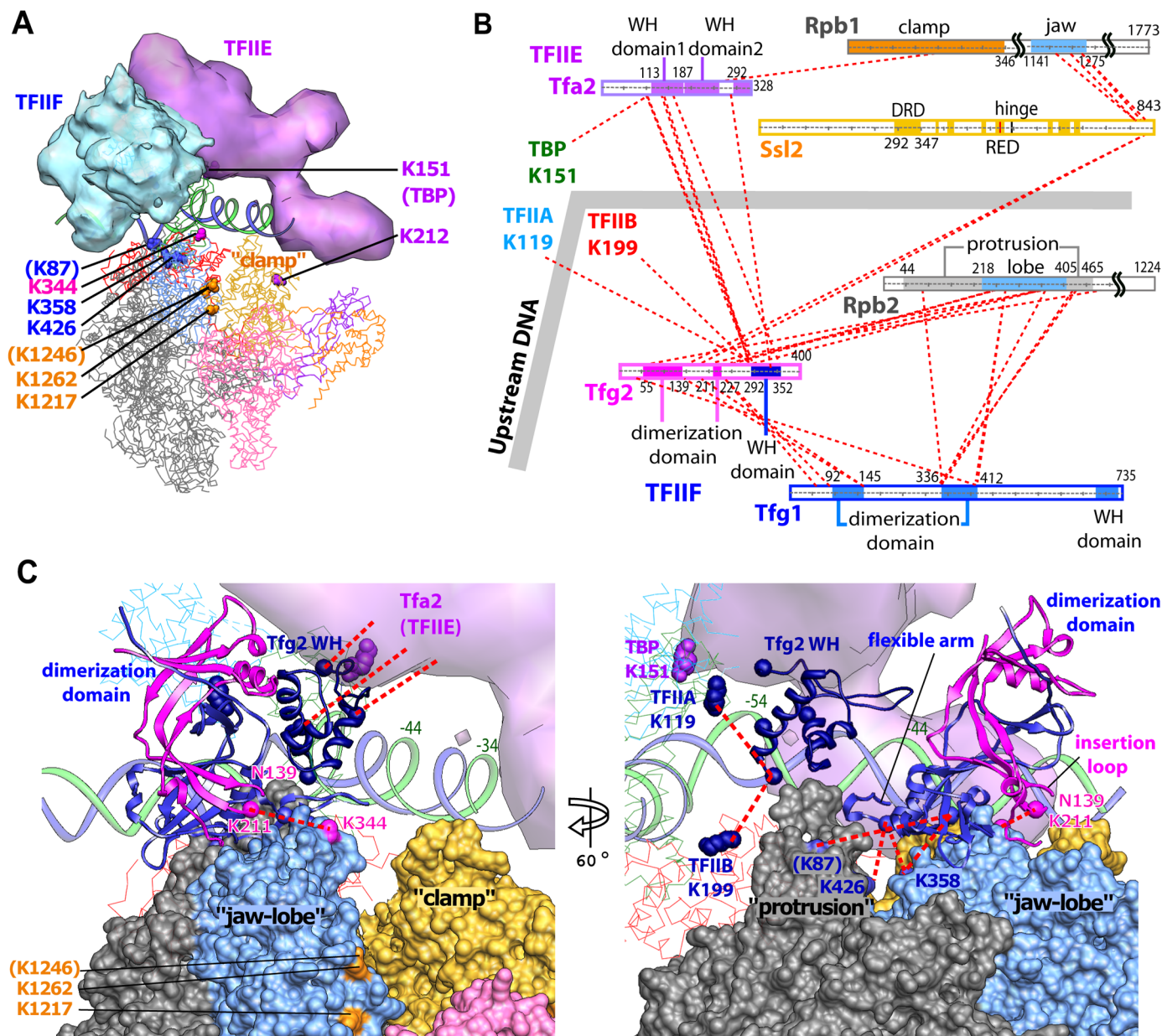


Fig. 4. Spatial restraints from XL-MS: domains of TFIIF. (A) Front view (same as Fig. 1A) of the PIC showing only the EM densities for TFIIF (light blue) and TFIIE (light purple). Pol II domains (jaw-lobe, light blue; clamp, yellow; core, gray; shelf, magenta), TFIIA (cyan), TFIIB (red), and TBP (green) are represented as backbone traces. Lysine residues of pol II and one of TBP that form cross-links to TFIIE and TFIIF are marked by van de Waals spheres and colored according to the subunit to which they are cross-linked (Tfg1, blue; Tfg2, magenta; TFIIE, purple; Ssl2, orange). If the cross-linked residue is absent from the model, then the closest structured residue (up to three residues away in the amino acid sequence) is shown in

parentheses. **(B)** Schematic representation of cross-links (red dashed lines) involving Tfg1 and Tfg2, whose primary structures are depicted as boxes, with solid colors for regions of conserved folds. Only intersubunit cross-links are shown. **(C)** Close-up view of models of the Tfg1-Tfg2 dimerization domain (Tfg1, blue; Tfg2, magenta) and the WH domain of Tfg2 (dark blue) oriented on the basis of the cross-links indicated (red dashed lines). Pol II is shown in surface representation with the same colors as in (A). The Tfg2 yeast-specific insertion loop (residues 139 to 211) also cross-links to the C-terminal region of Ssl2, located near K1217, 1246, and 1262 of Rpb1.

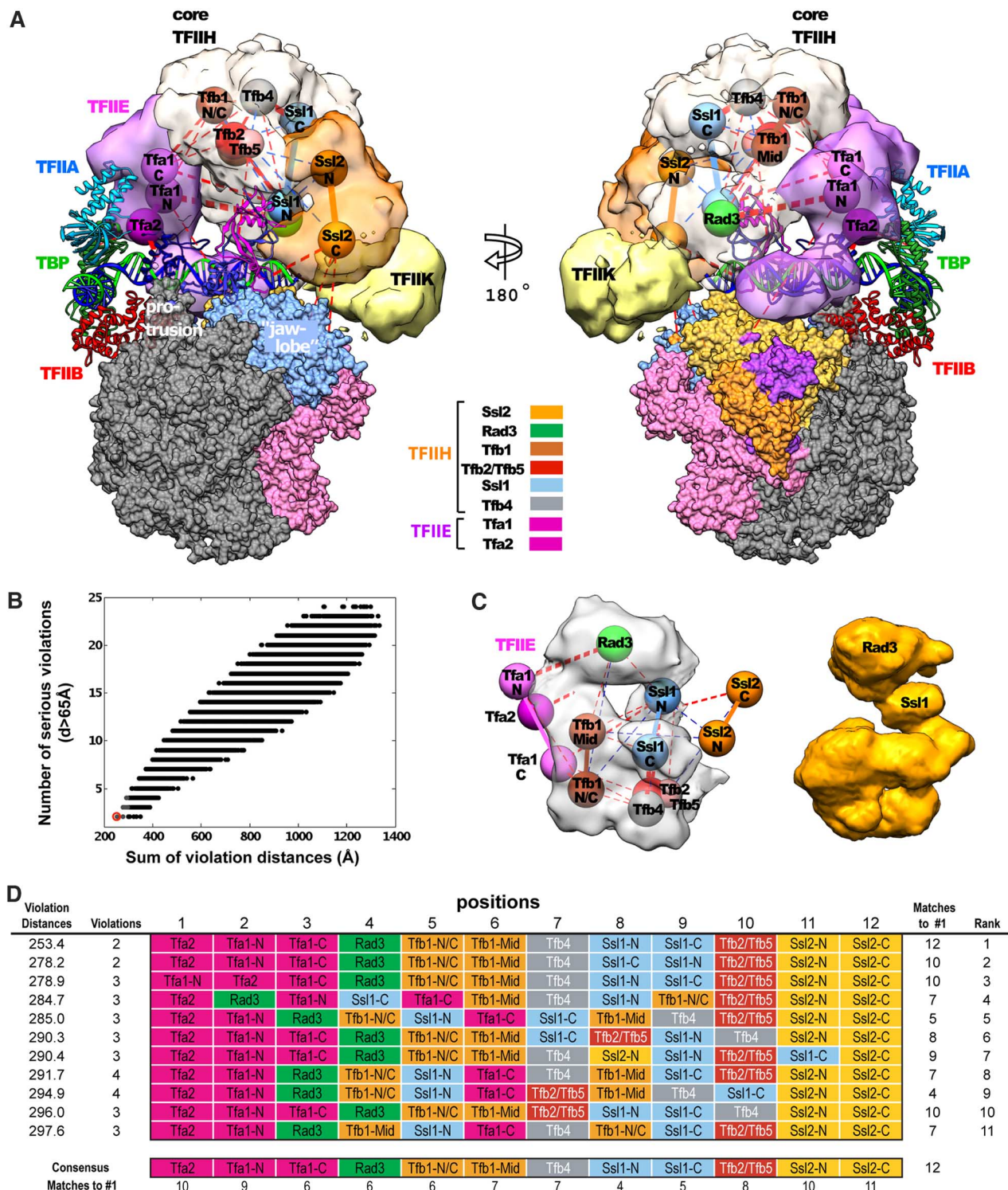


Fig. 5. Combination of XL-MS and cryo-EM: TFIIE and TFIIH. (A) Side views of PIC showing EM densities attributed to TFIIE (light purple) and TFIIH (core TFIIH, gray; Ssl2, orange; TFIIF, light yellow). Spheres for TFIIE, core TFIIH, and Ssl2 subunits are labeled according to the model that best fits the XL-MS data. Solid lines connect spheres belonging to the same subunit. Red dashed lines indicate intersubunit cross-links in the PIC, with thicknesses proportional to the number of cross-links observed. Blue dashed lines indicate cross-links in holo-TFIIH only. The EM density for TFIIE is omitted for clarity. Other elements of the PIC are represented as in Fig. 4C. (B) Fit of various models of the subunit locations (scatter) to XL-MS data. Two measures of fit are plotted for each model: the number of cross-linked spheres that are more than 65 Å apart (y axis) and

the sum of distances in excess of 40 Å between cross-linked spheres (x axis). The best-fitting model (red circle) is shown in (A). (C) Comparison of the density for core TFIIH (left) from (A) with the reconstructed volume of core TFIIH from EM of 2D crystals in stain (right) (18). Locations of TFIIE (three purple spheres) and Ssl2 (two orange spheres) from (A) are shown on the left as well. (D) Listing of the 11 best fitting models [gray scatter in (B)], detailing for each the assignment of the subunits to the 12 fixed positions in the electron density. Each row represents a different model, with the best-fitting model (A) listed at the top. The most frequently occurring subunit at each position (the consensus) is identical to that of the best-fitting model. The strength of the consensus (bottom row) is indicative of the confidence of subunit assignment.

(transcription bubble) in two ways, by the introduction of untwisting strain (by the helicase) and by positioning promoter DNA.

Untwisting strain is distributed throughout the DNA above the pol II cleft, so melting may occur at any point, but only a melted region adjacent to TFIIB is stabilized by binding to pol II. The reason is again the rigidity of duplex DNA, and the requirement for a sharp bend adjacent to TFIIB to penetrate the pol II cleft. A single strand of DNA must extend from the point of contact with TFIIB, ~13 bp downstream of the TATA box (36, 37), through the binding site for the transcription bubble in pol II. TFIIB may

also interact with the single strand to stabilize the bubble (14).

Our conclusions are based on results from both cryo-EM and XL-MS, which served to validate one another: Segmentation and labeling of electron density, based on fitting pol II and other known structures, was consistent with all but three of 266 cross-links observed. Our PIC structure is also consistent with partial structural information from x-ray crystallography [pol II-TFIIB (12–14), pol II-TFIIS (31), TFIIA-TBP-TFIIB-DNA (15, 32), and Tfb2-Tfb5 (38)], from nuclear magnetic resonance [Tfb1-Tfa1 (39) and Tfa2-DNA (40)], and from EM [core and holo TFIIF (18, 20)]. This

consistency provides cross-validation, both supporting our PIC structure and establishing the relevance of the partial structural information. Further consistency with the results of FeBABE cleavage mapping of complexes formed in yeast nuclear extract (34) was mentioned above; the locations of proteins along the DNA in our PIC structure and those determined with FeBABE cleavage differ by no more than 5 bp. Our PIC structure also agrees with results of protein-DNA cross-linking in a reconstituted human transcription system (35); positions of TFIIE and TFIIF differ between the two studies by ~20 and 10 bp. The location of Ssl2 in our structure, ~30 bp downstream from the TATA box (Fig. 3, B and C), supports the proposal, made on the basis of previous DNA-protein cross-linking analysis, that helicase action torques the DNA to introduce untwisting strain and thereby to promote melting at a distance (35).

While this manuscript was in preparation, Nogales and coworkers reported EM structures of human PICs (41). The Nogales structures were produced from presumptive partial complexes through the alignment of EM images to a structure of pol II as a search model, so they unavoidably include the pol II structure. Beyond pol II and two GTF polypeptides, TBP and TFIIB, there is little resemblance between the Nogales structures and ours (Fig. 6, A and B, and fig. S14). The Nogales structures have no G-lobe (Fig. 6B); contain very little density for TFIIE (30% of expected) and TFIIF (20%); are inconsistent with the majority of the intermolecular cross-links involving TFIIE, TFIIF, and TFIIF that we observed; and show a very different DNA path from our structure (Fig. 7). In our view, the Nogales structures do not reveal a complete PIC, but only a complex of pol II with TBP, TFIIB, and DNA, for two reasons: First, partial complexes are unstable, so TFIIE and TFIIF failed to bind or were lost; and second, because image processing was performed with the pol II structure as a search model, conformational flexibility was neglected, and only proteins rigidly bound to pol II were observed. Differences from our complete PIC structure and the reasons for these differences are described in more detail in the supplementary materials.

The difference in DNA path between the Nogales structures and ours explains a difference

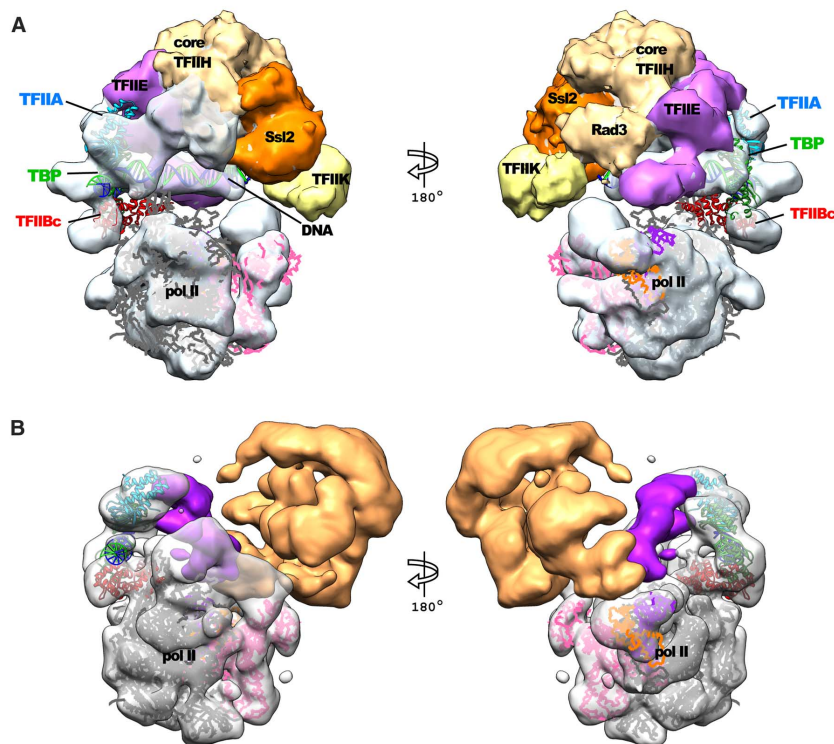
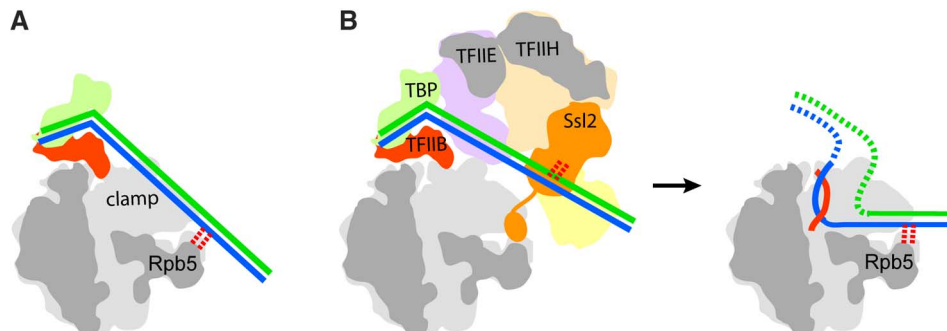


Fig. 6. Comparison of reconstructed volume of the PIC in this work to that of negatively stained human PIC. (A) Side views of the PIC from this work. Rotated by 60° (left) or -120° (right) relative to front view in Fig. 1A. (B) The reconstruction from negatively stained human PIC (41) is viewed in the same pol II orientation as in (A). The extra density due to TFIIF (light brown) is only weakly anchored to the remainder of the PIC. The center of mass of TFIIF is displaced by ~90 Å compared with the structure in (A). TFIIE (purple) is dissociated from TBP and TFIIB. Density due to DNA is absent.

Fig. 7. Comparison of DNA paths within PIC structures. (A) Cutaway view in schematic representation of cryo-EM structure of PIC lacking TFIIE and TFIIF (41). TFIIF is omitted for clarity. DNA is blue and green. The downstream DNA contacts (red dotted lines) the tip of Rpb5. (B) (Left) Cutaway view in schematic representation of the complete PIC in this study. TFIIF is omitted for clarity. The C-terminal region of Ssl2 (orange appendage at the bottom of Ssl2) binds residues within the pol II cleft, preventing entry of DNA, which is instead suspended above the cleft, where it interacts with Ssl2 (red dotted lines). (Right) Cutaway view for the transcribing complex based on the x-ray structure (11), and contact with Rpb5 in the cleft is indicated (red dotted lines). Nascent RNA is red.



between two reports of protein-DNA cross-linking of pol II-GTF-DNA complexes and suggests a role for the C-terminal domain of Ssl2; a high-frequency cross-link of Rpb5 to DNA downstream of the TATA box was identified for a PIC lacking TFIIF (42, 43), whereas no such cross-link was found for a complete PIC, but instead a cross-link of Ssl2 was observed at the same position (34). The trajectory of the DNA in the Nogales structures, penetrating the pol II cleft at the location of Rpb5, is thus explained by the absence of TFIIE and TFIIF, whereas the location of the DNA in our structure is due to the presence of TFIIF. The position of DNA in the Nogales structures would clash with the location of the C-terminal domain of Ssl2 in the cleft in our structure. We suggest that the site bound by DNA in the Nogales structure is blocked by the C-terminal domain of Ssl2; only after promoter melting and entry of the template strand at the upstream end of the pol II cleft does DNA displace the C-terminal domain of Ssl2 and occupy the downstream end of the cleft (Fig. 7B). Region 1.1 of $\sigma 70$ performs a similar role, binding in the cleft of bacterial RNA polymerase, preventing nonspecific DNA interaction, and dissociating upon open complex formation (44).

References and Notes

- R. C. Conaway, J. W. Conaway, General initiation factors for RNA polymerase II. *Annu. Rev. Biochem.* **62**, 161–190 (1993). doi: [10.1146/annurev.bi.62.070193.001113](#); pmid: [8352588](#)
- R. D. Kornberg, The molecular basis of eukaryotic transcription. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 12955–12961 (2007). doi: [10.1073/pnas.0704138104](#); pmid: [17670940](#)
- R. D. Kornberg, Mediator and the mechanism of transcriptional activation. *Trends Biochem. Sci.* **30**, 235–239 (2005). doi: [10.1016/j.tibs.2005.03.011](#); pmid: [15896740](#)
- S. K. Burley, R. G. Roeder, Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* **65**, 769–799 (1996). doi: [10.1146/annurev.bi.65.070196.004005](#); pmid: [8811195](#)
- S. Buratowski, S. Hahn, L. Guarente, P. A. Sharp, Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56**, 549–561 (1989). doi: [10.1016/0092-8674\(89\)90578-3](#); pmid: [2917366](#)
- S. N. Gudder, P. Sung, V. Bailly, L. Prakash, S. Prakash, RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. *Nature* **369**, 578–581 (1994). doi: [10.1038/369578a0](#); pmid: [8202161](#)
- W. J. Feaver, O. Gileadi, Y. Li, R. D. Kornberg, CTD kinase associated with yeast RNA polymerase II initiation factor b. *Cell* **67**, 1223–1230 (1991). doi: [10.1016/0092-8674\(91\)90298-D](#); pmid: [1836979](#)
- Y. J. Kim, S. Björklund, Y. Li, M. H. Sayre, R. D. Kornberg, A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**, 599–608 (1994). doi: [10.1016/0092-8674\(94\)90221-6](#); pmid: [8187178](#)
- P. Cramer *et al.*, Architecture of RNA polymerase II and implications for the transcription mechanism. *Science* **288**, 640–649 (2000). doi: [10.1126/science.288.5466.640](#); pmid: [10784442](#)
- P. Cramer, D. A. Bushnell, R. D. Kornberg, Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science* **292**, 1863–1876 (2001). doi: [10.1126/science.1059493](#); pmid: [11313498](#)
- A. L. Gnatt, P. Cramer, J. Fu, D. A. Bushnell, R. D. Kornberg, Structural basis of transcription: An RNA polymerase II elongation complex at 3.3 Å resolution. *Science* **292**, 1876–1882 (2001). doi: [10.1126/science.1059495](#); pmid: [11313499](#)
- D. A. Bushnell, K. D. Westover, R. E. Davis, R. D. Kornberg, Structural basis of transcription: An RNA polymerase II-TFIIB cocystal at 4.5 Å resolution. *Science* **303**, 983–988 (2004). doi: [10.1126/science.1090838](#); pmid: [14963322](#)
- D. Kostrewa *et al.*, RNA polymerase II-TFIIB structure and mechanism of transcription initiation. *Nature* **462**, 323–330 (2009). doi: [10.1038/nature08548](#); pmid: [19820686](#)
- X. Liu, D. A. Bushnell, D. Wang, G. Calero, R. D. Kornberg, Structure of an RNA polymerase II-TFIIB complex and the transcription initiation mechanism. *Science* **327**, 206–209 (2010). doi: [10.1126/science.1182015](#); pmid: [19965383](#)
- D. B. Nikolov *et al.*, Crystal structure of a TFIIB-TBP-TATA-element ternary complex. *Nature* **377**, 119–128 (1995). doi: [10.1038/377119a0](#); pmid: [7675079](#)
- W. H. Chung *et al.*, RNA polymerase II/TFIIF structure and conserved organization of the initiation complex. *Mol. Cell* **12**, 1003–1013 (2003). doi: [10.1016/S1097-2765\(03\)00387-3](#); pmid: [14580350](#)
- A. Jawhari *et al.*, Structure and oligomeric state of human transcription factor TFIIE. *EMBO Rep.* **7**, 500–505 (2006). pmid: [16547462](#)
- W. H. Chang, R. D. Kornberg, Electron crystal structure of the transcription factor and DNA repair complex, core TFIIF. *Cell* **102**, 609–613 (2000). doi: [10.1016/S0092-8674\(00\)00083-0](#); pmid: [11007479](#)
- P. Schultz *et al.*, Molecular structure of human TFIIF. *Cell* **102**, 599–607 (2000). doi: [10.1016/S0092-8674\(00\)00082-9](#); pmid: [11007478](#)
- B. J. Gibbons *et al.*, Subunit architecture of general transcription factor TFIIF. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1949–1954 (2012). doi: [10.1073/pnas.1105266109](#); pmid: [22308316](#)
- K. Murakami *et al.*, Tfb6, a previously unidentified subunit of the general transcription factor TFIIF, facilitates dissociation of Ssl2 helicase after transcription initiation. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 4816–4821 (2012). doi: [10.1073/pnas.1201448109](#); pmid: [22411836](#)
- K. Murakami *et al.*, Formation and fate of a complete 31-protein RNA polymerase II transcription preinitiation complex. *J. Biol. Chem.* **288**, 6325–6332 (2013). doi: [10.1074/jbc.M112.433623](#); pmid: [23303183](#)
- D. Elmlund, R. Davis, H. Elmlund, Ab initio structure determination from electron microscopic images of single molecules coexisting in different functional states. *Structure* **18**, 777–786 (2010). doi: [10.1016/j.str.2010.06.001](#); pmid: [20637414](#)
- D. Elmlund, H. Elmlund, SIMPLE: Software for ab initio reconstruction of heterogeneous single-particles. *J. Struct. Biol.* **180**, 420–427 (2012). doi: [10.1016/j.jsb.2012.07.010](#); pmid: [22902564](#)
- Z. A. Chen *et al.*, Architecture of the RNA polymerase II-TFIIF complex revealed by cross-linking and mass spectrometry. *EMBO J.* **29**, 717–726 (2010). doi: [10.1038/emboj.2009.401](#); pmid: [20094031](#)
- K. Lasker *et al.*, Molecular architecture of the 26S proteasome holoenzyme determined by an integrative approach. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1380–1387 (2012). doi: [10.1073/pnas.1120559109](#); pmid: [22307589](#)
- N. Kalisman, C. M. Adams, M. Levitt, Subunit order of eukaryotic Tric/CCT chaperonin by cross-linking, mass spectrometry, and combinatorial homology modeling. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 2884–2889 (2012). doi: [10.1073/pnas.1119472109](#); pmid: [22308438](#)
- D. M. Prather, E. Larschan, F. Winston, Evidence that the elongation factor TFIIS plays a role in transcription initiation at GAL1 in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **25**, 2650–2659 (2005). doi: [10.1128/MCB.25.7.2650-2659.2005](#); pmid: [15767671](#)
- B. Kim *et al.*, The transcription elongation factor TFIIS is a component of RNA polymerase II preinitiation complexes. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 16068–16073 (2007). doi: [10.1073/pnas.0704573104](#); pmid: [17913884](#)
- B. Kastner *et al.*, GraFix: Sample preparation for single-particle electron cryomicroscopy. *Nat. Methods* **5**, 53–55 (2008). doi: [10.1038/nmeth1139](#); pmid: [18157137](#)
- H. Kettenberger, K. J. Armache, P. Cramer, Complete RNA polymerase II elongation complex structure and its interactions with NTP and TFIIS. *Mol. Cell* **16**, 955–965 (2004). doi: [10.1016/j.molcel.2004.11.040](#); pmid: [15610738](#)
- S. Tan, Y. Hunziker, D. F. Sargent, T. J. Richmond, Crystal structure of a yeast TFIIB/TBP/DNA complex. *Nature* **381**, 127–134 (1996). doi: [10.1038/381127a0](#); pmid: [8610010](#)
- F. Gaiser, S. Tan, T. J. Richmond, Novel dimerization fold of RAP30/RAP74 in human TFIIF at 1.7 Å resolution. *J. Mol. Biol.* **302**, 1119–1127 (2000). doi: [10.1006/jmbi.2000.4110](#); pmid: [11183778](#)
- G. Miller, S. Hahn, A DNA-tethered cleavage probe reveals the path for promoter DNA in the yeast preinitiation complex. *Nat. Struct. Mol. Biol.* **13**, 603–610 (2006). doi: [10.1038/nsmb1117](#); pmid: [16819517](#)
- T. K. Kim, R. H. Ebricht, D. Reinberg, Mechanism of ATP-dependent promoter melting by transcription factor TFIIF. *Science* **288**, 1418–1421 (2000). doi: [10.1126/science.288.5470.1418](#); pmid: [10827951](#)
- F. C. Holstege, U. Fiedler, H. T. Timmers, Three transitions in the RNA polymerase II transcription complex during initiation. *EMBO J.* **16**, 7468–7480 (1997). doi: [10.1093/emboj/16.24.7468](#); pmid: [9405375](#)
- M. Pal, A. S. Ponticelli, D. S. Luse, The role of the transcription bubble and TFIIB in promoter clearance by RNA polymerase II. *Mol. Cell* **19**, 101–110 (2005). doi: [10.1016/j.molcel.2005.05.024](#); pmid: [15989968](#)
- D. E. Kainov, M. Vitorino, J. Cavarelli, A. Poterszman, J. M. Egly, Structural basis for group A trichothiodystrophy. *Nat. Struct. Mol. Biol.* **15**, 980–984 (2008). doi: [10.1038/nsmb.1478](#); pmid: [19172752](#)
- M. Okuda *et al.*, Structural insight into the TFIIE-TFIIF interaction: TFIIE and p53 share the binding region on TFIIF. *EMBO J.* **27**, 1161–1171 (2008). doi: [10.1038/emboj.2008.47](#); pmid: [18354501](#)
- M. Okuda *et al.*, Structure of the central core domain of TFIIEbeta with a novel double-stranded DNA-binding surface. *EMBO J.* **19**, 1346–1356 (2000). doi: [10.1093/emboj/19.6.1346](#); pmid: [10716934](#)
- Y. He, J. Fang, D. J. Taatjes, E. Nogales, Structural visualization of key steps in human transcription initiation. *Nature* **495**, 481–486 (2013). doi: [10.1038/nature11991](#); pmid: [23446344](#)
- T. K. Kim *et al.*, Trajectory of DNA in the RNA polymerase II transcription preinitiation complex. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12268–12273 (1997). doi: [10.1073/pnas.94.23.12268](#); pmid: [9356438](#)
- D. Forget, M. F. Langelier, C. Thérien, V. Trinh, B. Coulombe, Photo-cross-linking of a purified preinitiation complex reveals central roles for the RNA polymerase II mobile clamp and TFIIF in initiation mechanisms. *Mol. Cell. Biol.* **24**, 1122–1131 (2004). doi: [10.1128/MCB.24.3.1122-1131.2004](#); pmid: [14729958](#)
- V. Mekler *et al.*, Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase-promoter open complex. *Cell* **108**, 599–614 (2002). doi: [10.1016/S0092-8674\(02\)00667-0](#); pmid: [11893332](#)
- D. A. Bushnell, R. D. Kornberg, Complete, 12-subunit RNA polymerase II at 4.1-Å resolution: Implications for the initiation of transcription. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6969–6973 (2003). doi: [10.1073/pnas.1130601100](#); pmid: [12746498](#)

Acknowledgments: This research was supported by NIH grants GM49885 and AI21144 to R.D.K. and GM063817 to M.L. The EM map has been deposited in the EMDDataBank under accession code EMD-2394.

Supplementary Materials

www.sciencemag.org/content/342/6159/1238724/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S14
Tables S4 and S5
References (46–63)
Tables S1 to S3
Movies S1 to S3
Protein Data Bank File for the PIC Model

4 April 2013; accepted 5 September 2013
10.1126/science.1238724

Entangling Mechanical Motion with Microwave Fields

T. A. Palomaki,^{1,2*} J. D. Teufel,³ R. W. Simmonds,³ K. W. Lehnert^{1,2}

When two physical systems share the quantum property of entanglement, measurements of one system appear to determine the state of the other. This peculiar property is used in optical, atomic, and electrical systems in an effort to exceed classical bounds when processing information. We extended the domain of this quantum resource by entangling the motion of a macroscopic mechanical oscillator with a propagating electrical signal and by storing one half of the entangled state in the mechanical oscillator. This result demonstrates an essential requirement for using compact and low-loss micromechanical oscillators in a quantum processor, can be extended to sense forces beyond the standard quantum limit, and may enable tests of quantum theory.

During the development of quantum mechanics, entanglement was a troubling feature of the new theory (1). Although still counterintuitive, entanglement is now understood to be a resource; it must be generated, manipulated, and stored to process quantum information (2, 3). For example, entanglement is required to operate a quantum computer, to communicate with security guaranteed by physical laws, and in several strategies to circumvent the standard quantum limit of measurement (4–6). Extending entanglement into the domain of macroscopic mechanical oscillators would allow the properties of mechanical oscillators to be used in these quantum enhanced applications. Specifically, forces acting on the oscillator could be measured with improved precision; the oscillator could create a quantum coherent exchange between two otherwise noninteracting systems (7–9); and the relatively long lifetime of the oscillator could be used to store one half of an entangled pair of states, as required by quantum teleportation (10).

Creating an entangled state between a mechanical oscillator and another degree of freedom requires the ability to control the state of the oscillator at the level of individual mechanical quanta. Although quantum effects such as ponderomotive squeezing (11, 12) and radiation pressure shot noise (13) have been recently observed in optomechanical devices, currently, the most advanced control over the state of a mechanical oscillator has been realized in electromechanical devices by incorporating the oscillator in a superconducting circuit, either a superconducting qubit (14) or an inductor-capacitor (LC) resonator (15).

We used an electromechanical circuit to generate entanglement between the motion of a mechanical oscillator and a propagating microwave

field, following the scheme proposed in (16). In the entanglement process, one half of the entangled pair emerges as a microwave pulse in a transmission line, whereas the other half is stored in the mechanical oscillator. The state of the mechanical oscillator can be transferred on demand into a second microwave pulse (17). We demonstrate that the two microwave pulses are entangled by measuring their fields and showing that they are sufficiently correlated (18–20) to be in an inseparable (two-mode squeezed) state (21–23). Because the second microwave pulse encodes the state of the mechanical oscillator that was created during the entanglement process, the first microwave pulse was consequently entangled with the mechanical oscillator.

The interaction between the mechanical oscillator and microwave fields is created in an LC resonator where the capacitor is mechanically compliant. The fundamental mode of motion of the compliant capacitor, with resonance frequency Ω_m , is the harmonic oscillator that will be en-

tangled with microwave fields. Because the resonance frequency of the LC resonator ω_c changes with the position $\hat{x} = x_{zp}(\hat{b} + \hat{b}^\dagger)$ of the mechanical oscillator, the interaction between the LC resonator and the mechanical oscillator is described by the Hamiltonian $H_{\text{int}} = \hbar g_0(\hat{b} + \hat{b}^\dagger)\hat{a}^\dagger\hat{a}$, where $\hat{a}(\hat{a}^\dagger)$ is the annihilation(creation) operator for resonator photons, $\hat{b}(\hat{b}^\dagger)$ is the annihilation(creation) operator for mechanical phonons, $g_0 = x_{zp}(d\omega_c/dx)$, and x_{zp} is the oscillator's zero-point motion. The LC resonator is coupled to a nearby transmission line so that a microwave photon in the resonator evolves into a propagating photon in the transmission line at a rate κ_{ext} .

The electromechanical device combines a microfabricated vacuum-gap capacitor and spiral inductor into an LC resonator (Fig. 1A) (15). It is fabricated from superconducting aluminum and cooled in a cryostat to a temperature of $T < 20$ mK. In preliminary measurements of the type described in (15), we determined the electro-mechanical parameters of the device. The LC resonance frequency is $\omega_c \approx 2\pi \times 7.7$ GHz, and the mechanical oscillator resonates at $\Omega_m = 2\pi \times 10.34$ MHz. The LC resonator and oscillator are coupled at a rate $g_0 = 2\pi \times 200$ Hz. Energy decays from the LC resonator at a total rate $\kappa = \kappa_{\text{ext}} + \kappa_{\text{int}} = 2\pi \times 360$ kHz, where $\kappa_{\text{ext}} = 2\pi \times 300$ kHz is the coupling of the resonator to the transmission line, and κ_{int} is the rate at which energy is dissipated within the resonator. Last, the mechanical oscillator loses energy to its environment at a rate $\gamma_m = 2\pi \times 35$ Hz.

To control the state of the mechanical oscillator, we coupled it much more strongly to propagating microwave fields in the transmission line (via the LC resonator) than to its dissipative environment. This coupling was created by exciting the LC resonator with an intense microwave tone—a “pump” (Fig. 1, B and C). If the tone is applied at a frequency $\omega_r = \omega_c - \Omega_m$ (red-detuned),

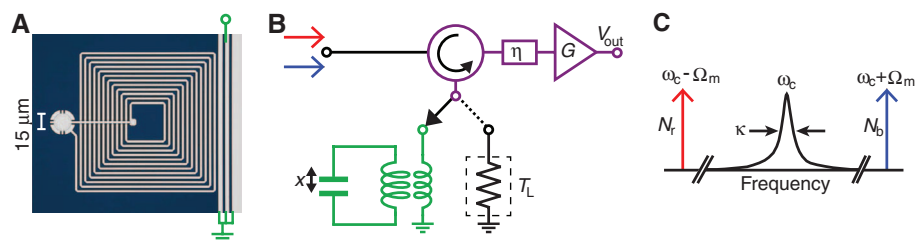


Fig. 1. Schematic description of the experiment. (A) A device image shows the aluminum (gray) and sapphire substrate (blue). The disk on the left side of the figure is the capacitor, and the spiral is the inductor. Electrical energy in the resulting LC resonator is coupled through mutual inductance to a microwave transmission line on the right edge of the figure. The center conductor of the transmission line is shorted to the adjacent aluminum (ground symbol). (B) A simplified circuit schematic shows that either the device (green) or a variable temperature T_L resistor is connected by a switch to the measurement apparatus (magenta), whose output V_{out} is proportional to the microwave fields at the input. With the switch connected to the load, we vary T_L , injecting known thermal noise into the measurement apparatus, and determine both the measurement gain G and quantum efficiency η . With the switch connected to the device, control pumps (red and blue arrows) excite the device. (C) A frequency domain representation of the experiment shows the response of the circuit (black) and the entangling (blue) and transfer (red) pumps detuned above or below ω_c by Ω_m .

¹JILA, National Institute of Standards and Technology and the University of Colorado, Boulder, CO 80309, USA. ²Department of Physics, University of Colorado, Boulder, CO 80309, USA. ³National Institute of Standards and Technology, Boulder, CO 80305, USA.

*Corresponding author. E-mail: tauno.palomaki@jila.colorado.edu

we refer to it as the transfer pump, and the interaction Hamiltonian is approximately

$$H_{bs} = \hbar g_0 \sqrt{N_r(t)} (\hat{d} \hat{b}^\dagger + \hat{b} \hat{d}^\dagger) \quad (1)$$

where $\hat{d} = \hat{a} - \sqrt{N_r}$ is the deviation of the resonator field from the average coherent amplitude, and N_r is the strength of the pump, expressed as the average number of resonator photons induced by the pump (24). This beam-splitter Hamiltonian implies that the mechanical oscillator and propagating microwave fields in the transmission line coherently exchange their states at a rate $\Gamma_r = 4g_0^2 N_r / \kappa$ (16, 17). Because this rate can exceed the decoherence rate of the mechanical oscillator $\Gamma_r > \gamma_m k_B T / (\hbar \Omega_m)$, and because the propagating fields are naturally in pure quantum states, the

mechanical oscillator can be prepared in a low-entropy state with less than one phonon (15).

Although the beam-splitter Hamiltonian can transfer quantum states between the mechanical oscillator and propagating microwave fields, by itself it cannot entangle the mechanical oscillator and the microwave fields. But if the LC resonator is excited with a blue-detuned pump at $\omega_b = \omega_c + \Omega_m$ (entangling pump), the resulting interaction Hamiltonian is very different

$$H_{pdc} = \hbar g_0 \sqrt{N_b(t)} (\hat{d}^\dagger \hat{b}^\dagger + \hat{b} \hat{d}) \quad (2)$$

with N_b defined analogously to N_r . In this case, the Hamiltonian is a parametric down-conversion interaction, capable of generating phonons and photons in pairs. If this parametric down-conversion

acts on the oscillator's zero-point motion and microwave vacuum fluctuations, it will create a two-mode entangled state between the oscillator and a propagating microwave pulse. In order to create substantial entanglement, the rate at which entangled phonons and photons are generated must exceed the rate of mechanical decoherence $\Gamma_b = 4g_0^2 N_b / \kappa > \gamma_m k_B T / (\hbar \Omega_m)$ (16).

The protocol to generate entanglement between mechanical motion and microwave fields exploits both the beam-splitter and parametric down-conversion interaction (Fig. 2). We first applied a strong transfer pump that cools the mechanical oscillator so that it contains less than one phonon on average (15). We then turned off the transfer pump, waited 15 μ s, and applied the constant amplitude entangling pump for a time τ . The microwave field in the transmission line incident on the circuit was amplified (25) and reflected as a microwave pulse with a center frequency ω_c and an envelope rising exponentially at rate $\Gamma_b/2$ (16). From the pair-wise creation of photons and phonons described by Eq. 2, we expected that the mechanical oscillator's motion would be also amplified. After storing this amplified state of the mechanical oscillator for time τ_{del} , we converted it to a microwave pulse by applying the transfer pump a second time. The microwave pulse that emerges from the circuit also has a center frequency ω_c but an envelope that decays exponentially with rate $\Gamma_r/2$. Because we determine $\Gamma_r/2$, $\Gamma_b/2$, and ω_c in separate measurements, we can extract the state of the two microwave pulses expressed as quadrature amplitudes, X_b and Y_b for the first pulse and X_r and Y_r for the second (26), where X_r and Y_r encode the state of the mechanical oscillator just before the transfer pump is applied (17). We calibrated the quadrature amplitudes by injecting microwave vacuum noise into the measurement apparatus (Fig. 1B) and expressed the quadrature amplitudes in units of vacuum noise [a mode in its ground state would have variances $\text{Var}(X_r) = \text{Var}(Y_r) = 1/2$]. Last, by injecting a known amount of thermal noise into the measurement apparatus, we can determine its quantum efficiency $\eta = 0.20 \pm 0.01$ (26), where a noiseless measurement of a single quadrature would yield $\eta = 1$. Because we measure both quadratures of the microwave pulses simultaneously, quantum noise limits $\eta < 0.5$ (26).

To understand the statistical properties of the two microwave pulses generated from the protocol (Fig. 2B), we first considered them separately. We repeated the protocol 10,000 times for a given value of Γ_b and found the two, two-dimensional distributions of measurement outcomes (X_b , Y_b) and (X_r , Y_r), which are both Gaussian (Fig. 3, A and B). We found the variances $U_r = \text{Var}(X_r) + \text{Var}(Y_r)$ and $U_b = \text{Var}(X_b) + \text{Var}(Y_b)$ for the measurement outcomes. To understand how these variances change with $\Gamma_b = 4g_0^2 N_b / \kappa$, we repeated the protocol with several different values of N_b . We observed that U_r and U_b grow approximately exponentially with Γ_b , demonstrating that the parametric down-conversion process amplifies

Fig. 2. Entanglement protocol. (A) A timing diagram illustrates the cooling, entanglement, and transfer phases of the protocol. (B) The plot shows V_{out} versus time in one instance of the protocol. Although not discernable from the figure, both pulses have a center frequency of ω_c that can be well-resolved from the control pumps.

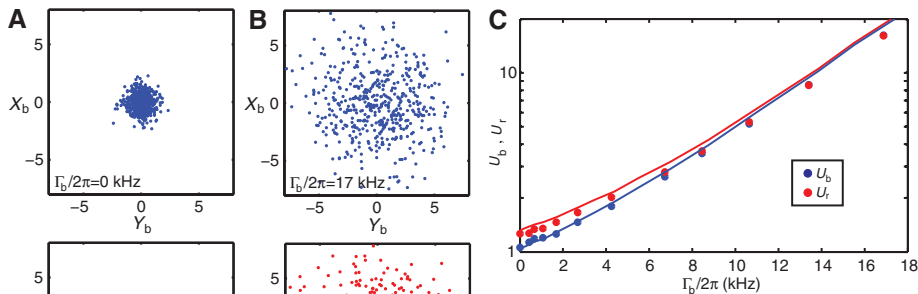
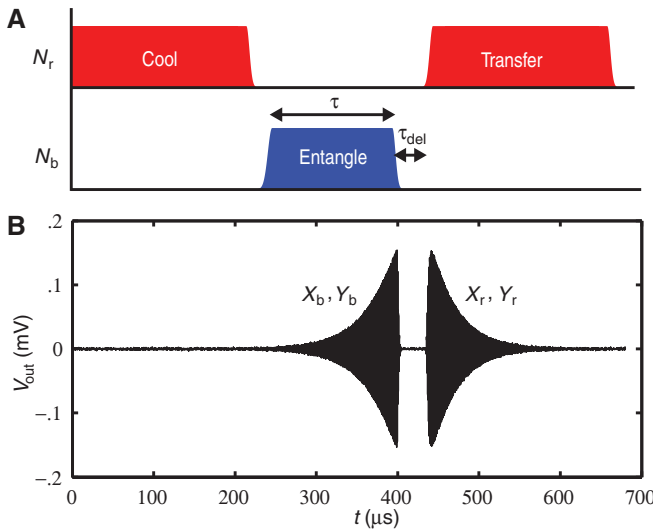


Fig. 3. Amplifying vacuum fluctuations. (A and B) Each point shows the measured state (X_b , Y_b) or (X_r , Y_r) of the two microwave pulses created in a single instance of the protocol. The plots show measurements for 500 repetitions of the protocol for (A) $\Gamma_b/2\pi = 0$ kHz and (B) $\Gamma_b/2\pi = 17$ kHz, both with $\tau = 35.5$ μ s, $\tau_{del} = 10$ μ s, and $\Gamma_r/2\pi = 11$ kHz. The four plots are each consistent with a two-dimensional Gaussian distribution with zero mean. (C) The variance of the phase-space distribution for the first pulse (blue points) and second pulse (red points) are plotted versus Γ_b , with $\tau = 35.5$ μ s, $\tau_{del} = 10$ μ s, and $\Gamma_r/2\pi = 11$ kHz. The points are generated from 10,000 repetitions of the protocol at each value of Γ_b . The lines show the variances of the two pulses (colors correspond) predicted by a numerical integration of the equations of motion, with all parameters independently determined. In addition, the prediction uses the fact that the mechanical oscillator is initially cooled to have a total variance of $2.0 \pm 0.2 \times x_{zp}^2$ and that the LC resonator has a total noise with variance $1.24 \pm 0.04 \times$ vacuum variance (15).

$\Gamma_r/2\pi = 11$ kHz. The four plots are each consistent with a two-dimensional Gaussian distribution with zero mean. (C) The variance of the phase-space distribution for the first pulse (blue points) and second pulse (red points) are plotted versus Γ_b , with $\tau = 35.5$ μ s, $\tau_{del} = 10$ μ s, and $\Gamma_r/2\pi = 11$ kHz. The points are generated from 10,000 repetitions of the protocol at each value of Γ_b . The lines show the variances of the two pulses (colors correspond) predicted by a numerical integration of the equations of motion, with all parameters independently determined. In addition, the prediction uses the fact that the mechanical oscillator is initially cooled to have a total variance of $2.0 \pm 0.2 \times x_{zp}^2$ and that the LC resonator has a total noise with variance $1.24 \pm 0.04 \times$ vacuum variance (15).

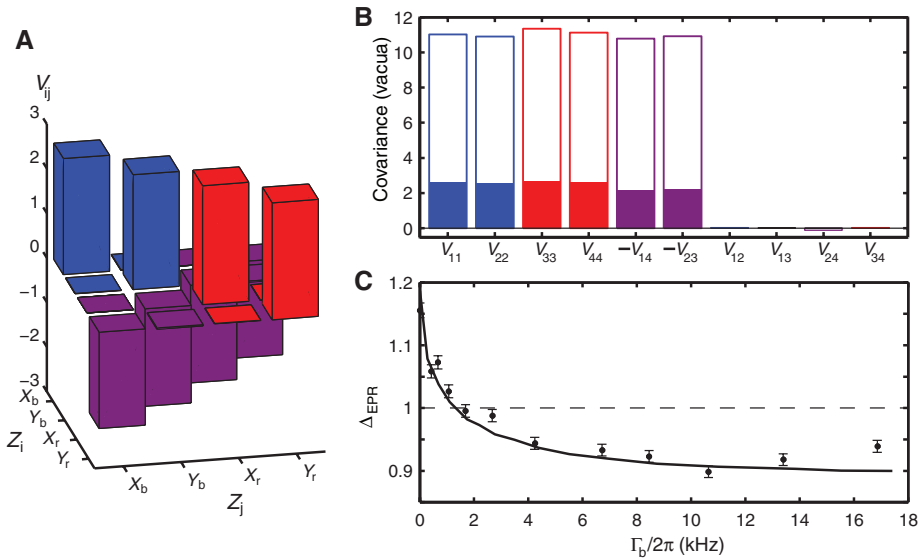


Fig. 4. Correlations and inseparability. (A) We plot the covariance matrix for the protocol with $\Gamma_b/2\pi = 10.6$ kHz, $\tau = 35.5$ μ s, and $\tau_{\text{del}} = 10$ μ s. The elements are averaged over 10,000 repetitions. (B) The 10 independent elements of the covariance matrix are shown (solid bars) for the same data as (A), but with the intermode correlations inverted, $-V_{14} = -(\langle X_b Y_r \rangle - \langle X_b \rangle \langle Y_r \rangle)$ and $-V_{23} = -(\langle Y_b X_r \rangle - \langle Y_b \rangle \langle X_r \rangle)$, to aid comparison with the diagonal elements. We can infer the matrix elements (open bars) that describe the state of the two microwave pulses using the measurement efficiency η . (C) The measured Δ_{EPR} (points) versus Γ_b , determined from the same data used in Fig. 3C, falls below vacuum fluctuations (dashed line). The expectation (solid line) also uses parameters identical to those of Fig. 3C.

the fluctuations in the microwave fields and in the mechanical oscillator's motion with power gain of approximately $\exp(\Gamma_b \tau)$ (Fig. 3C), as predicted by the equations of motion of the electromechanical device (26). When the modes are considered separately, the amplification associated with the parametric down-conversion process appears to have substantially increased the entropy of the two modes.

In order to reveal that the entropy has not increased appreciably (27) and that the two modes are entangled, we studied the correlations between them. These correlations are revealed by calculating the Gaussian part of the two-mode state, which is completely specified by the 10 independent elements of the 4×4 covariance matrix $V_{ij} \equiv \langle Z_i Z_j \rangle - \langle Z_i \rangle \langle Z_j \rangle$, where $Z_i \in \{X_b, Y_b, X_r, Y_r\}$, and the brackets denote an average over the 10,000 measurements. As seen in Fig. 4, A and B, the entropy in the two modes measured separately, apparent as a large variance of the diagonal elements V_{ii} , is almost completely compensated by the intermode covariances, V_{14} and V_{23} . To distinguish purely classical correlations from a quantum mechanically entangled state, we determined the inseparability parameter (16, 22, 23, 28)

$$\Delta_{\text{EPR}} = \frac{1}{2} [\text{Var}(X_r + Y_b) + \text{Var}(X_b + Y_r)] \quad (3)$$

which would be $\Delta_{\text{EPR}} = 1$ if the two pulses were both in their vacuum state. For $\Delta_{\text{EPR}} < 1$, the two pulses are entangled. Because the second microwave pulse encodes the state of the mechanical oscillator created during the entangling operation, the state of the first microwave pulse and the

mechanical oscillator are entangled if the two microwave pulses are entangled. In Fig. 4C, we plot the measured value Δ_{EPR} versus Γ_b . When the strength of the entangling pump is weak ($\Gamma_b/2\pi < 2$ kHz), there is no entanglement; however, Δ_{EPR} falls below vacuum when $\Gamma_b/2\pi > 3$ kHz, with a minimum of $\Delta_{\text{EPR}} = 0.90 \pm 0.01$, clearly demonstrating entanglement. The measured inseparability parameter is consistent with the value predicted by the equations of motion for $\Gamma_b/2\pi < 14$ kHz.

Because the measurement apparatus does not have perfect quantum efficiency, the measured value of Δ_{EPR} is closer to 1 than it would have been if $\eta = 1$. By measuring η , we can infer the covariance matrix and the inseparability parameter Δ_{EPR} that characterizes the state of the microwave pulses (rather than the measurement of the pulses) using $\Delta_{\text{EPR}} = \eta \Delta_{\text{EPR}} + (1 - \eta)$. The elements of the inferred covariance matrix are shown as the open bars in Fig. 4B, yielding $\Delta_{\text{EPR}} = 0.50 \pm 0.06$.

To quantify the entanglement, we found the logarithmic negativity E_N (29, 30) for both the measured state and the inferred state. This quantity bounds the number of maximally entangled Bell pairs that can be distilled from the state (31). For Gaussian states, it can be calculated as $E_N = -\log_2(4\lambda_1 \lambda_2)/2$, where λ_1 and λ_2 are the two smallest eigenvalues of the covariance matrix (30). For the measured state $E_N = 0.16 \pm 0.02$, whereas for the inferred state $E_N = 1.1 \pm 0.2$, meaning each instance of the protocol generates one Bell pair worth of entanglement. Because we calibrate the measurements using itinerant microwave fields, any noise process or loss that affects

the mechanical oscillator or the LC resonator will diminish the inferred entanglement rather than simply reduce its visibility through η . The most important such process is the decoherence of the mechanical oscillator because of its thermal environment. When τ_{del} exceeds the mechanical coherence time $\hbar\Omega_m/(\gamma_m k_B T) \cong 120$ μ s, sufficient time has elapsed for one phonon to enter the mechanical oscillator from the environment, and indeed, we found no entanglement $\Delta_{\text{EPR}} > 1$. Yet, the mechanical oscillator has stored a true quantum feature for at least $\tau_{\text{del}} = 10$ μ s (Fig. 4).

In contrast to other recent methods to generate continuous variable entanglement with purely microwave fields (18–20), one half of our entangled pair is stored in a long-lived memory (17) and then retrieved from the memory on demand. Indeed, the fact that half of the entangled state is stored in the mechanical oscillator highlights the potential for these oscillators to serve as quantum memories for microwave signals. Our measurements further demonstrate that mechanical oscillators enable quantum applications not just by storing information, but also by generating quantum mechanically useful entangled states (32). In addition to their use in quantum information processing and precision force measurements, these entangled states of mechanical motion have even been considered as a means to test quantum theory in an unexplored domain (33).

References and Notes

1. A. Einstein, B. Podolsky, N. Rosen, *Phys. Rev.* **47**, 777–780 (1935).
2. S. L. Braunstein, P. van Loock, *Rev. Mod. Phys.* **77**, 513–577 (2005).
3. C. Weedbrook *et al.*, *Rev. Mod. Phys.* **84**, 621–669 (2012).
4. M. Tsang, C. M. Caves, *Phys. Rev. X* **2**, 031016 (2012).
5. M. J. Woolley, A. A. Clerk, *Phys. Rev. A* **87**, 063846 (2013).
6. W. Wasilewski *et al.*, *Phys. Rev. Lett.* **104**, 133601 (2010).
7. Sh. Barzanjeh, M. Abdi, G. J. Milburn, P. Tombesi, D. Vitali, *Phys. Rev. Lett.* **109**, 130503 (2012).
8. L. Tian, *Phys. Rev. Lett.* **110**, 233602 (2013).
9. Y. D. Wang, A. A. Clerk, *Phys. Rev. Lett.* **110**, 253601 (2013).
10. A. Furusawa *et al.*, *Science* **282**, 706–709 (1998).
11. D. W. C. Brooks *et al.*, *Nature* **488**, 476–480 (2012).
12. A. H. Safavi-Naeini *et al.*, *Nature* **500**, 185–189 (2013).
13. T. P. Purdy, R. W. Peterson, C. A. Regal, *Science* **339**, 801–804 (2013).
14. A. D. O'Connell *et al.*, *Nature* **464**, 697–703 (2010).
15. J. D. Teufel *et al.*, *Nature* **475**, 359–363 (2011).
16. S. G. Hofer, W. Wieczorek, M. Aspelmeyer, K. Hammerer, *Phys. Rev. A* **84**, 052327 (2011).
17. T. A. Palomaki, J. W. Harlow, J. D. Teufel, R. W. Simmonds, K. W. Lehnert, *Nature* **495**, 210–214 (2013).
18. E. P. Menzel *et al.*, *Phys. Rev. Lett.* **109**, 250502 (2012).
19. E. Flurin, N. Roch, F. Mallet, M. H. Devoret, B. Huard, *Phys. Rev. Lett.* **109**, 183901 (2012).
20. C. Eichler *et al.*, *Phys. Rev. Lett.* **107**, 113601 (2011).
21. Z. Y. Ou, S. F. Pereira, H. J. Kimble, K. C. Peng, *Phys. Rev. Lett.* **68**, 3663–3666 (1992).
22. L. M. Duan, G. Giedke, J. I. Cirac, P. Zoller, *Phys. Rev. Lett.* **84**, 2722–2725 (2000).
23. R. Simon, *Phys. Rev. Lett.* **84**, 2726–2729 (2000).
24. J. Zhang, K. Peng, S. L. Braunstein, *Phys. Rev. A* **68**, 013808 (2003).
25. F. Massel *et al.*, *Nature* **480**, 351–354 (2011).
26. Materials and methods are available as supplementary materials on Science Online.

27. A. Serafini, F. Illuminati, S. De Siena, *J. Phys. B* **37**, L21–L28 (2004).
 28. W. P. Bowen, R. Schnabel, P. K. Lam, T. C. Ralph, *Phys. Rev. A* **69**, 012304 (2004).
 29. G. Vidal, R. F. Werner, *Phys. Rev. A* **65**, 032314 (2002).
 30. M. M. Wolf, J. Eisert, M. B. Plenio, *Phys. Rev. Lett.* **90**, 047904 (2003).
 31. K. Audenaert, M. B. Plenio, J. Eisert, *Phys. Rev. Lett.* **90**, 027901 (2003).
 32. S. G. Hofer, D. V. Vasilyev, M. Aspelmeier, K. Hammerer, *Phys. Rev. Lett.* **111**, 170404 (2013).
 33. Y. Chen, *J. Phys. B* **46**, 104001 (2013).

Acknowledgments: We thank H.-S. Ku, J. Kerckhoff, J. Harlow, and S. Clancy for helpful discussions. This material is based on work supported by the National Science Foundation under grant 1125844, the Defense Advanced Research Projects Agency QuEST program, and the Gordon and Betty Moore Foundation through grant GBMF3503 to K.W.L.

Supplementary Materials

www.sciencemag.org/content/342/6159/710/suppl/DC1
 Materials and Methods
 Figs. S1 to S4
 References (34–39)

12 August 2013; accepted 18 September 2013
 Published online 3 October 2013;
 10.1126/science.1244563

A Thermoelectric Heat Engine with Ultracold Atoms

Jean-Philippe Brantut,¹ Charles Grenier,² Jakob Meineke,^{1*} David Stadler,¹ Sebastian Krinner,¹ Corinna Kollath,³ Tilman Esslinger,^{1†} Antoine Georges^{2,4,5}

Thermoelectric effects, such as the generation of a particle current by a temperature gradient, have their origin in a reversible coupling between heat and particle flows. These effects are fundamental probes for materials and have applications to cooling and power generation. Here, we demonstrate thermoelectricity in a fermionic cold atoms channel in the ballistic and diffusive regimes, connected to two reservoirs. We show that the magnitude of the effect and the efficiency of energy conversion can be optimized by controlling the geometry or disorder strength. Our observations are in quantitative agreement with a theoretical model based on the Landauer-Büttiker formalism. Our device provides a controllable model system to explore mechanisms of energy conversion and realizes a cold atom-based heat engine.

Heat and charge transport in materials are often coupled processes (1). This coupling leads to thermoelectric effects: A temperature gradient may lead to a voltage drop, or vice versa. These effects are important for probing elementary excitations in materials and have practical applications to refrigeration and power generation from waste-heat recovery (2, 3). Recently, there has also been interest in thermoelectric effects in nano- and molecular-scale electronic devices (4, 5). The progress in modeling solid-state physics with cold atoms (6, 7) raises the question of whether thermoelectricity can be observed in such a controlled setting (8–10), where setups analogous to mesoscopic transport devices have been realized (11–13). Although the thermodynamic interplay between thermal and density modes has been seen in a second sound experiment (14) and studied in the theory of the fountain effect (15, 16), thermoelectric transport has so far not been investigated.

Here, we demonstrate a cold atoms device in which a temperature bias generates a neutral atom current, analogous to a charge current in conductors. A schematic view of the experimental setup is shown in Fig. 1A. It is based on our previous work (13, 17). Initially we prepare N_{tot} =

$3.1(4) \times 10^5$ weakly interacting ^6Li atoms at a temperature of 250(9) nK in an elongated trap, where the Fermi temperature of the cloud is $T_F = 931(44)$ nK. A repulsive laser beam (not shown) having a nodal line at its center separates the cloud into two identical reservoirs connected by a quasi-two-dimensional channel. Tuning the power of the beam allows one to adjust the trap frequency ν_z in the channel up to 10 kHz. We then raise a gate potential in the channel, preventing any exchange between the reservoirs. A heating beam heats the left reservoir in a controlled way, increasing its temperature by typically 200 nK. Afterwards, the gate potential is removed abruptly, allowing heat and particle exchange between the reservoirs for a variable time. Before determining the particle number and temperature in each reservoir, they are separated by raising the gate potential. The power of the laser beam creating the channel is then ramped to zero in 400 ms, and each reservoir is left to equilibrate independently for 100 ms (18).

We measure the temperatures T_h (T_c) and atom number N_h (N_c) in the hot (cold) reservoir using absorption images and reconstruct the time evolution of the number imbalance $\Delta N = N_c - N_h$ and temperature bias $\Delta T = T_c - T_h$. Figure 1, B and C, show typical results. The temperatures equilibrate fast, similar to the equilibration of atom numbers observed in the case of pure atomic flow (13). In contrast, the atom number difference in Fig. 1C starts at zero and shows an initial build-up driven by the thermopower of the channel. At later times, when temperatures have equilibrated, the imbalance results in a chemical potential bias, which brings the imbalance back to zero. This transient atomic current, created in response to a

temperature gradient, is the fingerprint of the intrinsic thermoelectric power of the channel. Because the compressible cloud in the hot reservoir expands, one naïvely expects an initial particle flow from the cold, denser side to the hot one. In contrast, we observe the opposite effect: a net particle current initially directed from the hot to the cold side.

To explain quantitatively our observations, we model the channel with its conductance G , thermal conductance G_T , and thermopower α_{ch} , which are the linear response coefficients for the current and entropy current to temperature and chemical potential bias. In this framework, the particle and entropy currents flowing in the channel are given by

$$\begin{pmatrix} I_N \\ I_S \end{pmatrix} = -G \begin{pmatrix} 1 & \alpha_{\text{ch}} \\ \alpha_{\text{ch}} & L + \alpha_{\text{ch}}^2 \end{pmatrix} \begin{pmatrix} \mu_c - \mu_h \\ T_c - T_h \end{pmatrix} \quad (1)$$

In this expression, $I_N = \partial \Delta N / \partial t$ and $I_S = \partial \Delta S / \partial t$ are the particle and entropy currents, with

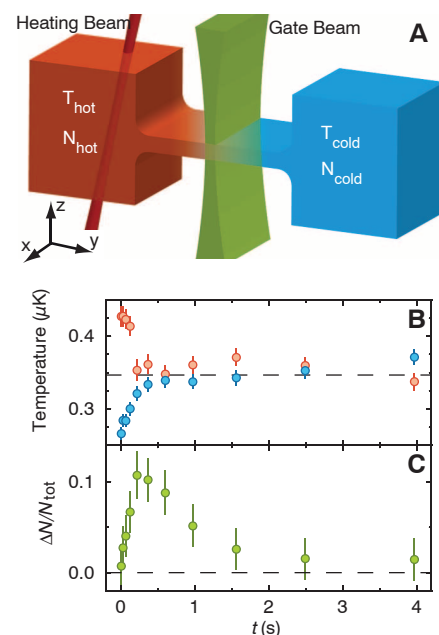


Fig. 1. Concept of the experiment. (A) A quasi-two-dimensional channel connects two atomic reservoirs. A gate beam intersects with the channel and blocks particle and heat transport. A heating beam traverses the left reservoir and heats it in a controlled way. **(B)** T_h (red) and T_c (blue) as a function of time. Dashed line: $\bar{T}$ at the initial time. **(C)** $\Delta N/N_{\text{tot}}$ as a function of time. In the channel, ν_z was set to 3.5 kHz with a disorder of average strength 542 nK (see text).

¹Institute for Quantum Electronics, ETH Zürich, CH-8093 Zürich, Switzerland. ²Centre de Physique Théorique, École Polytechnique, CNRS, 91128 Palaiseau cedex, France. ³HISKP, Universität Bonn, Nussallee 14-16, D-53115 Bonn, Germany. ⁴Collège de France, 11 place Marcelin Berthelot, 75005 Paris, France. ⁵DPMC-MaNEP, Université de Genève, CH-1211 Genève, Switzerland.

*Present address: Philips Technologie GmbH, Röntgenstraße 24-26, D-22335 Hamburg.

†Corresponding author. E-mail: esslinger@phys.ethz.ch

$\Delta S = S_c - S_h$. Further, μ_h and μ_c are the chemical potentials of the hot and cold reservoirs; $L = G_T/TG$ is the Lorenz number (19) of the channel, which measures the ratio of entropy to particle current noise at equilibrium; and $\bar{T} = (T_h + T_c)/2$. Combining Eq. 1 with the thermodynamics of the reservoirs leads to the equation for the time evolution of ΔN and ΔT :

$$\tau_0 \frac{d}{dt} \begin{pmatrix} \Delta N \\ \Delta T \end{pmatrix} = - \begin{pmatrix} 1 & -\kappa(\alpha_r - \alpha_{ch}) \\ -\frac{\alpha_r - \alpha_{ch}}{\ell\kappa} & \frac{L + (\alpha_r - \alpha_{ch})^2}{\ell} \end{pmatrix} \begin{pmatrix} \Delta N \\ \Delta T \end{pmatrix} \quad (2)$$

Here, $\kappa = \frac{\partial N}{\partial \mu}|_T$, $C_N = T \frac{\partial S}{\partial T}|_N$, and $\alpha_r = \frac{\partial S}{\partial N}|_T$ are, respectively, the compressibility, specific heat, and dilatation coefficient of each reservoir, calculated at the average temperature $\bar{T}$ and particle number $(N_c + N_h)/2$. $\ell = \frac{C_N}{\kappa\bar{T}}$ is an analog of the Lorenz number for the reservoirs, measuring the relative magnitude of thermal fluctuations of entropy and atom number, and $\tau_0 = \kappa G^{-1}$ is the particle transport time scale, analogous to a capacitor's discharge time (13).

Equation 2 shows that the thermoelectric response results from the competition between the entropy transported through the channel, described by α_{ch} , and the entropy created by removing one atom from one reservoir and adding it to the other, described by α_r . In our system, τ_0 (typically ~ 0.5 s) is longer than both the longest oscillation period in the trap and the expected collision time in the reservoirs (~ 50 ms). Hence, we assume that reservoirs maintain thermal equilibrium at any time, and calculate G , G_T , and α_{ch} using the Landauer-Büttiker formalism (18, 20, 21). Within this description, the thermopower of the channel is controlled by the energy dependence of the transmission rate. Because the rate increases with energy, there is an excess current of high-energy particles flowing from hot to cold over low-energy particles flowing from cold to hot.

The energy dependence of the transmission coefficient, and hence α_{ch} , can be modified by changing the geometry of the channel (22). We measured the transient imbalance and the temperature evolution for various confinements in a ballistic channel. Two examples for $v_z = 3.5$ and 9.3 kHz are presented in Fig. 2, A and B. The evolution of both ΔN and ΔT is faithfully described by the theoretical model [eq. S14 in (18)], which does not involve any adjustable parameter. The dynamics of both temperature and atom-number evolution (Fig. 2C) is slowing down with increasing v_z , as expected because the number of conducting modes is reduced. The experimental values for τ_0 are extracted from fitting the experimental data with the theoretical model with τ_0 as the only free parameter (18). They agree well with the ab initio theoretical predictions and with independent measurements performed with a pure particle number imbalance (18).

The amplitude of the transient imbalance also increases with stronger confinement. We define the thermoelectric response $\mathcal{R} = (\Delta N/N_{\text{tot}})/(\Delta T_0/T_F)$, where ΔT_0 is the initial temperature difference. In Fig. 2D, we present the maximum thermoelectric response $\mathcal{R}_{\text{max}}$, which we fitted using the theoretical model where τ_0 was left as a free parameter. It displays an approximately linear increase with v_z , well reproduced by the theory (18).

We now investigate the effects of disorder on thermoelectricity. We project a blue detuned laser speckle pattern on the channel, which creates a random potential of average value and standard deviation $\bar{V}$ (18). This leads to a random, isotropic distribution of hills and wells in the plane of the channel. Figure 3A presents the time evolution of $\Delta N/N_{\text{tot}}$ for increasing disorder, for fixed $v_z = 3.5$ kHz. First, we observe that the time scale increases: The resistance increases as the channel crosses over from ballistic to diffusive. In addition to this slowdown, $\mathcal{R}_{\text{max}}$ increases from 0.17(8) without disorder to 0.55(16) for a strong disorder of 1.1 μK [still below the percolation threshold of the disorder of ~ 1.8 μK (23)].

For the strongest disorder, we observe (Fig. 3B) that the thermoelectric response saturates, while the time scale τ_0 keeps increasing, indicating the continuous increase of resistance with disorder. To further investigate this point, we performed experiments for a fixed confinement in the channel of 4.95 kHz, and several large disorder strengths ranging from 542 to 1220 nK. In this regime, the full data set collapses to a single curve (Fig. 3C), provided the time axis is rescaled by the time scale τ_0 extracted from an independent atomic conduction experiment (13, 18).

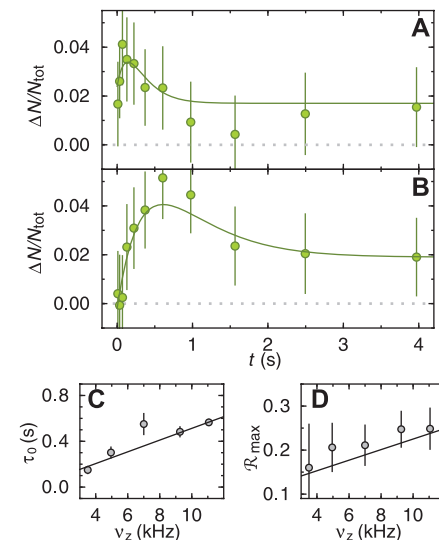


Fig. 2. Thermoelectricity in a ballistic channel. (A and B) Time evolution of $\Delta N/N_{\text{tot}}$ for $v_z = 3.5$ kHz (A) and $v_z = 9.3$ kHz (B). (C and D) Time scale τ_0 and maximum thermoelectric response $\mathcal{R}_{\text{max}}$ as a function of confinement. Symbols represent the values fitted from experiment, and the solid line is the theoretical prediction [see text and (18)].

This scaling property comes from the thermopower being a ratio of two linear response coefficients. Thus, it does not depend on the actual value of transparency, as the resistance does, but only on the way the transparency varies with energy. The variation is fixed by the diffusive nature of the transport process, leading to a universal thermoelectric response independent of the scattering time. This confirms that thermopower is less sensitive than resistance to the details of the conductor, a fact widely used in condensed-matter physics (24).

The effect of disorder can be described by extending our theoretical model, introducing an energy-dependent transparency of the constriction [eq. S10 in (18)]. This transparency involves the energy-dependent mean free path in the channel, which is the product of the particle velocity and scattering time. At strong disorder, the scattering time is assumed to be energy independent. When expressed as a function of t/τ_0 , a unique theoretical scaling curve independent of $\bar{V}$ is predicted for the thermoelectric response, which agrees well with the experimental data (Fig. 3C), with τ_0 as the only adjustable parameter. The dependence of τ_0 on disorder strength, fitted to the strongest disorder data only, together with the requirement that resistance interpolates between

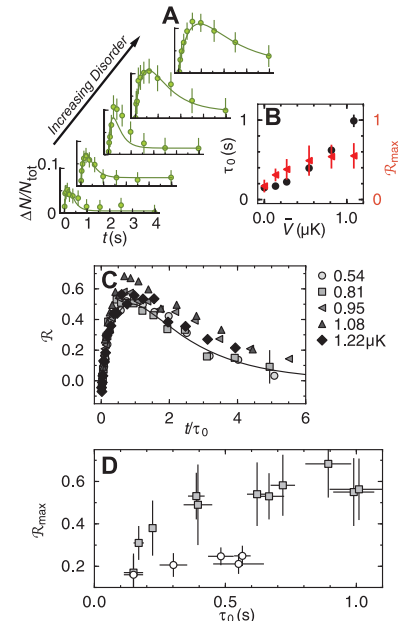


Fig. 3. Thermoelectricity in the ballistic-to-diffusive crossover. (A) Time evolution of $\Delta N/N_{\text{tot}}$ for increasing disorder strength ($\bar{V} = 0.14, 0.27, 0.54, 0.81$, and 1.08 μK), for a fixed confinement of $v_z = 3.5$ kHz. Solid lines: theory (18). (B) Fitted time scale τ_0 (black circles) and $\mathcal{R}_{\text{max}}$ (red triangles) as a function of disorder strength for the data set shown in (A). (C) Rescaled time evolution of $\mathcal{R}$ (see text) in the regime of strong disorder from $\bar{V} = 542$ nK to 1220 nK and fixed $v_z = 4.95$ kHz. A representative error bar is shown for late times. Black line: theoretical calculation (18). (D) $\mathcal{R}_{\text{max}}$ versus time scale τ_0 for the diffusive (gray squares) and ballistic case (open circles).

Ohm's law and ballistic transport from strong to weak disorder, yields a full model for the ballistic-to-diffusive crossover (18). The resulting theoretical curves for the transient particle imbalance accurately describe the data over the entire crossover (Fig. 3A) without extra fitting parameters.

Confinement and disorder are two independent ways of influencing the thermoelectric properties. To compare their respective merits, we display $\mathcal{R}_{\max}$ as a function of τ_0 in Fig. 3D. We find that disorder is more efficient than confinement to increase $\mathcal{R}_{\max}$. For the largest time scales, we observe an approximately threefold increase in $\mathcal{R}_{\max}$ in the diffusive case compared to the ballistic one. This is because thermoelectricity is due to the asymmetry between the motion of low- and high-energy particles. In the ballistic case, it originates from an increase in particle velocity and density of states with energy. For diffusive motion with a given scattering time, an extra dependence emerges: The rate at which particles with a given energy will cross the disordered region is itself an increasing function of energy, as it is proportional to the mean free path.

In the experiment, a controlled exchange of heat between a hot and a cold reservoir is used to produce a directed current, i.e., work. This motivates an analysis in terms of heat engines. To do so, we evaluate the work, efficiency, and power of the process. The area enclosed in the $\mu - N$ plane (Fig. 4A) represents the work $W = 1/2 \int_0^\infty dt (\mu_c - \mu_h)(t) I_N(t)$ produced during the evolution, which we evaluate from $N_{c,h}$ and $T_{c,h}$. Similarly, we evaluate the irreversible heat associated to the transport process $Q = -1/2 \int_0^\infty dt (T_c - T_h)(t) I_S(t)$ [eq. S20 in (18)].

We introduce the relative efficiency $\eta = W/Q$ (2, 18). For a reversible (Carnot) process, $\eta = 1$. We find that η is largest in the strongly disordered regime, where the thermoelectric response is largest (Fig. 4, B and C). However, the efficiency of any heat engine is expected to increase as the

dynamics slows down, because the thermodynamic processes become closer to reversibility. Therefore, a complementary criterion to evaluate the merits of the various thermoelectric configurations is the cycle-averaged power (25), estimated by W/τ_0 . Whereas the power is constant for the ballistic case (Fig. 4D), it shows a maximum in the diffusive case (Fig. 4E) and decreases for the largest disorder, because the increase in work is overcompensated by the slowdown. This is in qualitative agreement with the theory [eq. S23 in (18)]. We attribute the discrepancy with experiments to a small, finite imbalance offset not taken into account in the calculation.

We now focus on the channel, independently of the reservoirs. We use the transport coefficients extracted with our model to estimate the dimensionless figure of merit $ZT = \alpha_{\text{ch}}^2/L$ (2) of the channel (Fig. 4, F and G). This number is related to the efficiency achievable with a channel operating at maximum power and is used as a criterion for engineering thermoelectric devices (26). For the largest thermoelectric response observed, we infer $ZT = 2.4$, which is as large as the best values observed in any solid-state material (3). This is partly because our setup also allows one to explore thermoelectricity in the regime where phonons are absent and the ratio of temperature to Fermi temperature is large. This is not the case in conventional metals, which have a small ZT . However, in materials of current interest for thermoelectricity, such as strongly correlated “bad metals” (27), the relevant scale is the effective degeneracy temperature of quasiparticles T_F^* , so that T/T_F^* can be large.

We have demonstrated thermoelectric effects in quantum gases and shown that thermopower is a sensitive observable in this context. Our technique can be straightforwardly generalized to interacting systems where thermoelectric properties are of fundamental interest (27–30). The reversed

operation of our device leads in principle to cooling by the Peltier effect. This may be useful to cool quantum gases to low entropy, which is needed to explore strongly correlated fermions in lattices.

References and Notes

1. L. Onsager, *Phys. Rev.* **37**, 405–426 (1931).
2. H. J. Goldsmid, *Introduction to Thermoelectricity*, Springer Series in Materials Science (Springer, Dordrecht, Netherlands, 2009).
3. G. J. Snyder, E. S. Toberer, *Nat. Mater.* **7**, 105–114 (2008).
4. O. Entin-Wohlman, Y. Imry, A. Aharony, *Phys. Rev. B* **82**, 115314 (2010).
5. R. Sánchez, M. Büttiker, *Phys. Rev. B* **83**, 085428 (2011).
6. T. Esslinger, *Annu. Rev. Condens. Matter Phys.* **1**, 129–152 (2010).
7. I. Bloch, J. Dalibard, S. Nascimbene, *Nat. Phys.* **8**, 267–276 (2012).
8. C. Grenier, C. Kollath, A. Georges, <http://arxiv.org/abs/1209.3942> (2012).
9. H. Kim, D. A. Huse, *Phys. Rev. A* **86**, 053607 (2012).
10. C. H. Wong, H. T. C. Stoof, R. A. Duine, *Phys. Rev. A* **85**, 063613 (2012).
11. M. Albiez et al., *Phys. Rev. Lett.* **95**, 010402 (2005).
12. A. Ramanathan et al., *Phys. Rev. Lett.* **106**, 130401 (2011).
13. J.-P. Brantut, J. Meineke, D. Stadler, S. Krinner, T. Esslinger, *Science* **337**, 1069–1071 (2012).
14. L. A. Sidorenkov et al., *Nature* **498**, 78–81 (2013).
15. D. J. Papoular, G. Ferrari, L. P. Pitaevskii, S. Stringari, *Phys. Rev. Lett.* **109**, 084501 (2012).
16. T. Karpiuk, B. Grémaud, C. Miniatura, M. Gajda, *Phys. Rev. A* **86**, 033619 (2012).
17. D. Stadler, S. Krinner, J. Meineke, J.-P. Brantut, T. Esslinger, *Nature* **491**, 736–739 (2012).
18. Materials and methods are available as supplementary materials on Science Online.
19. N. W. Ashcroft, N. D. Mermin, *Solid State Physics* (Brooks Cole, Philadelphia, 1976).
20. Y. Imry, R. Landauer, *Rev. Mod. Phys.* **71**, S306–S312 (1999).
21. M. Büttiker, *IBM J. Res. Develop.* **32**, 317–334 (1988).
22. J. Heremans, M. Dresselhaus, in *Nanomaterials Handbook*, Y. Gogotsi, Ed. (CRC Press, Boca Raton, FL, 2006), chap. 27, pp. 739–770.
23. L. Pezzé et al., *New J. Phys.* **13**, 095015 (2011).
24. A. Mokashi et al., *Phys. Rev. Lett.* **109**, 096405 (2012).
25. H. B. Callen, *Thermodynamics* (Wiley, New York, 1960).
26. F. J. DiSalvo, *Science* **285**, 703–706 (1999).
27. I. Terasaki, *J. Appl. Phys.* **110**, 053705 (2011).
28. K. Behnia, D. Jaccard, J. Flouquet, *J. Phys. Condens. Matter* **16**, 5187–5198 (2004).
29. X. Zhang, C.-L. Hung, S.-K. Tung, N. Gemelke, C. Chin, *New J. Phys.* **13**, 045011 (2011).
30. T. Micklitz, A. Levchenko, A. Rosch, *Phys. Rev. Lett.* **109**, 036405 (2012).

Acknowledgments: We acknowledge fruitful discussions with J. Blatter, M. Büttiker, D. Papoular, and S. Stringari. We acknowledge funding from the Swiss National Science Foundation, National Centres of Competence in Research Materials with Novel Electronic Properties (MaNEP) and Quantum Science and Technology (QSIT), the European Research Council Project SQMS, the FP7 Project Nanodesigning of Atomic and Molecular Quantum Matter (NAME-QUAM), the ETH Zurich Schrödinger chair, the Defense Advanced Research Projects Agency–OLE program, Agence Nationale de la Recherche (Far From Equilibrium Quantum Systems), and ETH Zurich. J.-P.B. is partially supported by the European Union through a Marie Curie Fellowship. The data presented in this paper are available upon request to T.E.

Supplementary Materials

www.sciencemag.org/content/342/6159/713/suppl/DC1
Materials and Methods
Figs. S1 to S4
References (31–37)

24 June 2013; accepted 2 October 2013
Published online 24 October 2013;
10.1126/science.1242308

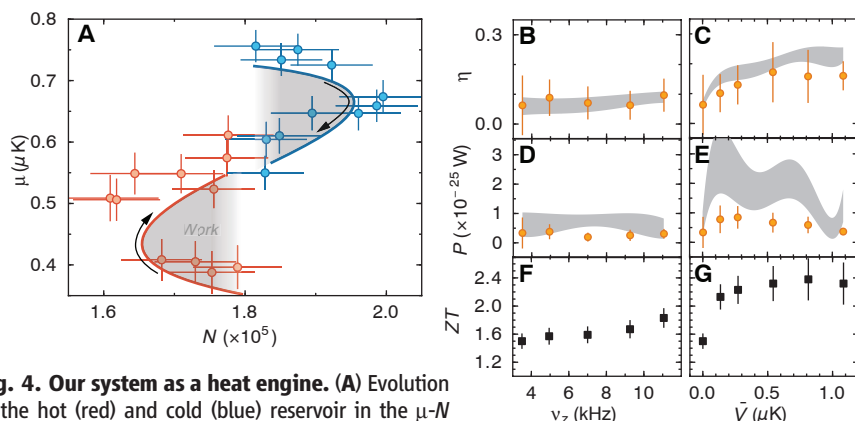


Fig. 4. Our system as a heat engine. (A) Evolution of the hot (red) and cold (blue) reservoir in the $\mu - N$ plane for $v_z = 3.5$ kHz and $\bar{V} = 542$ nK. Symbols: experiments; solid lines: theory (18). The black arrows indicate the direction of time, and the sum of the enclosed areas yields the total work. (B and D) Efficiency and power of the channel in the ballistic case, as a function of confinement. (C and E) The same quantities as a function of disorder strength for $v_z = 3.5$ kHz. Orange symbols: experiments; gray area: theory (theoretical error bars estimated from the uncertainties on the input parameters) (18). (F and G) Dimensionless figure of merit ZT as a function of confinement and disorder for ballistic and diffusive channels, respectively.

Visualization and Quantification of Electrochemical and Mechanical Degradation in Li Ion Batteries

Martin Ebner,¹ Federica Marone,² Marco Stampanoni,^{2,3} Vanessa Wood^{1*}

High-energy-density materials that undergo conversion and/or alloying reactions hold promise for next-generation lithium (Li) ion batteries. However, these materials experience substantial volume change during electrochemical operation, which causes mechanical fracture of the material and structural disintegration of the electrode, leading to capacity loss. In this work, we use x-ray tomography during battery operation to visualize and quantify the origins and evolution of electrochemical and mechanical degradation. Tomography provides the time-resolved, three-dimensional chemical composition and morphology within individual particles and throughout the electrode. In the model material tin(II) oxide, we witness distributions in onset and rate of core-shell lithiation, crack initiation and growth along preexisting defects, and irreversible distortion of the electrode, highlighting tomography as a tool to guide the development of durable materials and strain-tolerant electrodes.

Volume change of the active material during battery operation is the primary cause of short life in lithium ion batteries containing high-energy-density materials that undergo conversion and/or alloying reactions (1, 2). This class of materials—which includes pure metals, their alloys, oxides, fluorides, sulfates, nitrides, phosphates, and hydrides—offers up to an order of magnitude increase in specific capacity compared with the intercalation compounds that are used in commercial lithium ion batteries today (1). This potential has spurred considerable activities in material synthesis (1–6) and characterization (7–12), as well as theoretical studies and modeling efforts (13–16). However, due to the complexity of the interactions between electrochemistry and mechanical deformation (17–19), no comprehensive framework for rational material design exists to date.

An experimental technique providing quantitative chemical and morphological insight with three-dimensional (3D) spatial and temporal resolution that could advance the development of such a framework is needed. In situ x-ray diffraction (XRD) and Mössbauer spectroscopy quantify crystal structure evolution, whereas acoustic emission and dilatometry can be used to monitor fracture and expansion of entire electrodes (20–22). However, none of these approaches is able to directly relate the observed behavior of the ensemble to effects at the single-particle scale. On the other hand, techniques that enable visualization of single particles during electrochemical cycling—such as in situ transmission electron microscopy, scanning electron microscopy (SEM), and atomic force microscopy—are surface-sensitive techniques that cannot probe into the bulk of realistic porous

battery electrodes (7, 8, 23, 24). X-ray transmission microscopy has enabled observation of active particles, including Sn and SnO (9–11), as well as chemical phase mapping when used in combination with x-ray absorption near-edge structure spectroscopy (12). However, during electrochemical cycling, the 2D nature of these studies did not allow for quantitative assessment of particle phase evolution, volume expansion, or fracture.

Here, we show that synchrotron radiation x-ray tomographic microscopy (SRXTM) is a tool to simultaneously visualize and quantify the factors that affect battery performance. In SRXTM, monochromatic x-rays are directed onto the sample under investigation, which is rotated through 180° (Fig. 1A). 3D microstructure representations are then calculated numerically from recorded projection images by a tomographic reconstruction algorithm (25, 26). Previously, ex situ SRXTM and x-ray nanotomography have been used to characterize porous lithium ion battery electrodes (27, 28). We fabricated an electrochemical cell, compatible with SRXTM, to study electrodes during battery operation (Fig. 1B and fig. S1F) in a realistic environment (29). The cell is designed to be x-ray-transparent, to offer mechanical stability against vibrations during acquisition (to prevent reconstruction artifacts), and to provide good sealing and light pressure on the electrode for stable electrochemistry.

To demonstrate the complex interplay between electrochemistry and mechanical degradation in batteries, we opt to study tin(II) oxide (SnO) as a representative material that undergoes a conversion reaction and subsequent alloying with lithium (20, 30–32). We select a SnO synthesis route that offers size and shape control to create uniform particles, as shown in the SEM image in Fig. 1C (33). A porous electrode is fabricated from the SnO particles, carbon black, and polymeric binder and is inserted into the electrochemical cell.

Tomograms are recorded every 15 min during galvanostatic reduction (lithiation) at

110 mA-hour g^{−1} over a period of 12 hours and during oxidation (delithiation) at 167 mA-hour g^{−1} over 5 hours. Throughout the experiment, microstructural data of the entire electrode (diameter of 1.6 mm, initial thickness of 50 μm) are collected. The full electrode fits into the field of view to allow tracking of all particles during battery operation. To highlight the 3D nature of the collected data, we provide a rendering of a subsection of the electrode (Fig. 1D) corresponding to a volume of 333 by 33 by 50 μm³, or 1/25th of a full 3D data set. The voxel size is (0.65 μm)³, providing a true resolution of 2.0 μm, as determined from step profiles. Particles can be identified due to differences in x-ray attenuation coefficients (μ). A tomographic reconstruction without any image processing of an electrode cross section, recorded before electrochemical reduction, is shown in false-color in Fig. 1E. Yellow-red colors correspond to regions of high attenuation coefficients and can be associated with SnO particles, whereas the blue region corresponds to the minimally attenuating carbon black, polymer, and electrolyte phase that fills the space between the SnO particles. To obtain absolute values for the x-ray attenuation coefficient, a normalization procedure based on SnO and the background as internal standards is implemented, as discussed in the supplementary text.

Visualization and quantification of phase transformations within individual particles are possible due to changes in the attenuation coefficient, which is intimately linked to the composition and mass density of a material. In Fig. 1F, 12 snapshots selected from the 74 tomograms track two particles through their reactions. During reduction, we can qualitatively observe two consecutive processes consistent with the conversion reaction of SnO, in which nanosized Sn clusters form in a growing amorphous Li₂O (lithia) matrix, and the subsequent alloying reaction of the Sn clusters with lithium to form Li_xSn with 0 ≤ x ≤ 4.4 (20, 30–32). The Sn cluster alloying reaction is expected to traverse the equilibrium crystal phases present in the Li-Sn phase diagram at room temperature: Sn, Li_{0.4}Sn, LiSn, Li_{2.33}Sn, Li_{2.5}Sn, Li_{2.6}Sn, Li_{3.5}Sn, and finally, Li_{4.4}Sn. In Fig. 1F, we first observe a low-attenuating (yellow-green) front appear and progressively penetrate the particles, consuming the highly attenuating (yellow-red) phase. This core-shell process is the conversion reaction. Subsequently, the low-attenuating phase homogeneously transitions to an even lower-attenuating phase (dark green). This is the alloying reaction. During oxidation, we observe a homogeneous increase in attenuation coefficient from dark green to green-yellow. This process is consistent with the dealloying of the Li_xSn clusters in the lithia matrix. Accompanying the conversion and alloying reactions, we observe expansion of the particles as well as crack initiation and growth; during dealloying, we observe redensification of the particles. Because the voxel size is larger than the size and spacing of the Li_xSn clusters (both < 10 nm) in lithia (32), the attenuation coefficient obtained

¹Laboratory for Nanoelectronics, ETH Zurich, Gloriastrasse 35 CH-8092 Switzerland. ²Swiss Light Source, Paul Scherrer Institut, CH-5232 Switzerland. ³Institute for Biomedical Engineering, University of Zurich and ETH Zurich, Gloriastrasse 35 CH-8092 Switzerland.

*Corresponding author. E-mail: vwood@ethz.ch

by our technique represents a weighted average over the attenuation coefficients of the Li_xSn clusters and lithia matrix.

As indicated by the two particles shown in Fig. 1F and, more generally, in movie S1, particles exhibit a distribution in onset, rate, and completion of the core-shell reaction. To quantify the extent of the conversion reaction and enable direct comparison to electrochemical data, analysis must be performed considering a 3D electrode volume.

The chemical composition of the active particles within the electrode can be quantified during cell operation by analyzing the distribution of the x-ray attenuation coefficients. The leftmost peak in the attenuation coefficient histograms ($\mu = 0.4 \text{ cm}^{-1}$) from a central electrode subvolume (Fig. 2, A and B) corresponds to the carbon black, binder, and electrolyte phase, whereas the rightmost peak corresponds to the SnO particles

($\mu = 45 \text{ cm}^{-1}$). During electrochemical reduction (Fig. 2A), the SnO peak decreases in magnitude and vanishes after $\sim 500 \text{ mA}\cdot\text{hour g}^{-1}$. Simultaneously, a center peak associated with the formation of a lithia matrix studded with Sn clusters emerges at $\mu = 25 \text{ cm}^{-1}$ and then progressively shifts to 14 cm^{-1} as it increases in magnitude. Careful inspection of the SnO peak (close-up in fig. S2B) shows that it slowly shifts from 45 to 50 cm^{-1} , which can be explained by a disproportionation reaction of SnO to SnO_2 and Sn ($\mu = 49 \text{ cm}^{-1}$) (20). The shift toward higher attenuation coefficients can be seen visually in Fig. 1F and movie S1 as a shift to a deeper red color. This SnO_2 also undergoes a conversion reaction to form Li_xSn clusters in lithia that alloy to $\text{Li}_{4.4}\text{Sn}$ by the end of reduction (30).

During subsequent oxidation (Fig. 2B), the 14 cm^{-1} peak shifts back toward 30 cm^{-1} and

decreases in magnitude, consistent with the dealloying of Li_xSn and the contraction of the particles. The variation of the attenuation coefficient between the theoretical values for Sn in Li_2O ($\mu = 30 \text{ cm}^{-1}$) and $\text{Li}_{4.4}\text{Sn}$ in Li_2O ($\mu = 13 \text{ cm}^{-1}$) highlights the permanent existence of Li_xSn clusters within a lithia matrix. The temporal aspect of the phase evolution can be clearly seen by plotting the histograms from Fig. 2, A and B, as a density plot as a function of capacity, Q , in Fig. 2C. The disappearance of the SnO phase and emergence of the $\text{Li}_2\text{O} + \text{Sn}$ phase are clearly visible. We find a linear relation between the attenuation coefficient of the mixed phase and capacity, indicating a steady-state reaction. This bulk analysis sets the visual inspection of individual particles into perspective. For example, we conclude that the particle in the upper left of Fig. 1F lags behind the conversion of the ensemble, as it exhibits an unconverted core (red) at $729 \text{ mA}\cdot\text{hour g}^{-1}$. The particle in the lower right is more representative of the ensemble behavior.

As observed in Fig. 1F, the conversion and subsequent alloying reaction and the associated volume change are accompanied by crack formation and propagation in the active particles. Our choice of SnO enables us to identify the crystallographic orientation of particles in the tomography data and relate this to the observed cracking.

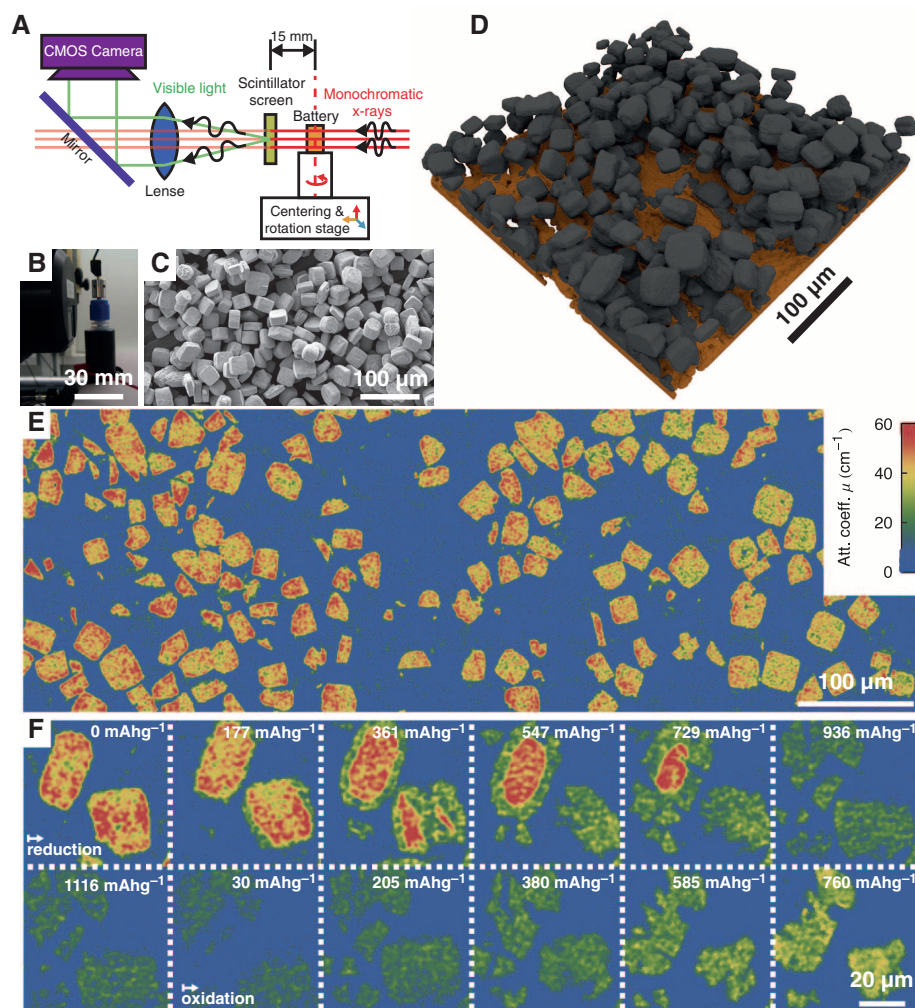


Fig. 1. X-ray tomography. (A) Sketch and (B) photograph of the x-ray tomography setup. CMOS, complementary metal-oxide semiconductor. (C) Scanning electron micrograph of SnO particles. (D) 3D visualization of x-ray tomograms recorded during battery operation. (E) Unprocessed cross-sectional tomogram showing individual SnO particles in the electrode with high resolution and good contrast against a low-attenuating carbon black, binder, and electrolyte phase. Att. coeff, attenuation coefficient. (F) A series of cross sections through two particles demonstrates a core-shell process, volume expansion, and particle fracture during the initial reduction and particle redensification during subsequent oxidation. $\text{mA}\cdot\text{h g}^{-1}$, milliamperes per gram.

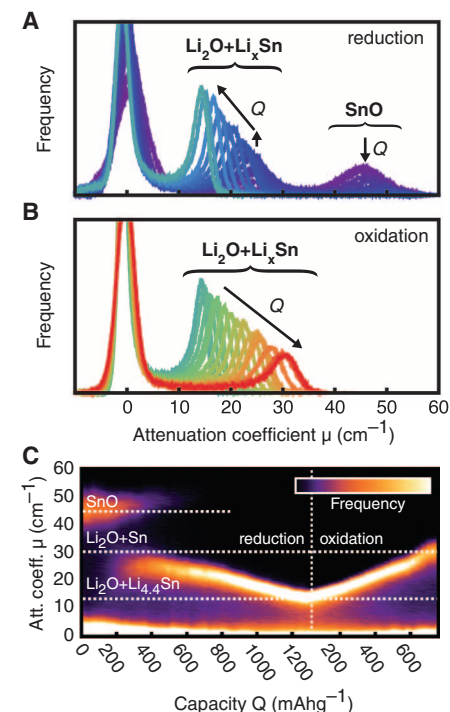


Fig. 2. Evolution of chemical composition. X-ray attenuation coefficient histograms during electrochemical (A) reduction and (B) oxidation. Color indicates temporal evolution. Q , capacity. (C) Attenuation coefficient distributions during electrochemical reduction and oxidation. Horizontal dotted white lines indicate theoretical attenuation coefficients for end members of the phase evolution.

XRD data and SEM analyses (provided in fig. S1) reveal pure SnO with tetragonal crystal structure and tetragonal particle shape. This coincidence of particle shape and crystal structure permits identification of the crystallographic orientation of tetragonal particles in the tomograms (33): A square face is associated with the (001) plane, whereas a rectangular face corresponds to the (100) or (010) plane. Figure 3, A and B, and movie S2, showing transverse and coronal cross sections through a particle during electrochemical reduction, reveal preferential cracking in the (001) plane originating at multiple sites along the [001] axis. We link these crack sites to grain boundaries that occur preferentially in (001) planes and are observable in the SEM images (fig. S1, A and B).

As highlighted by the white arrows in Fig. 3A, cracks appear sequentially at opposite edges of the particle. As observed by the absorption coefficient contrast in Fig. 3A and as indicated schematically in Fig. 3C, these cracks propagate inward, exposing fresh SnO surfaces to the electrolyte; these surfaces immediately undergo the conversion reaction. Volume expansion of this freshly exposed SnO contributes to the opening of the crack. This is highlighted by Fig. 3B, which shows that the SnO conversion in the crack plane occurs as a front diagonally traversing the particle. We speculate that as one crack is initiated and propagates, the stress in the vicinity of this crack is relaxed. Continuing reduction of the particle causes a buildup of stress at the opposite side of the particle, where propagation of the first crack does not alleviate the stress. Because cracks occur preferentially at grain boundaries, a new crack therefore initializes at the opposite side of the particle along a parallel grain boundary. These collective effects lead to the sequential delamination of (001) planes in the observed zig-zag geometry. The 3D renderings in Fig. 3D and movies S3 and S4 highlight the fact that this zig-zag delamination occurs in multiple particles. This observation that fracture is linked to crystallographic defects is consistent with the report of no fracture in defect-free SnO₂ nanowires (7). Studies of amorphous materials (which, by definition, do not have grains and grain boundaries) or nanostructured alloys would offer the opportunity to complete the picture of fracture as a function of material structure.

Synchrotron radiation x-ray tomographic microscopy further enables quantification of the volume change of the active particles in the entire electrode, a radiograph of which is shown in Fig. 4A. Details of the data processing are described in the supplementary text. Figure 4B shows the volume expansion calculated from our tomography data (gray circles) and the cell potential (purple line) as a function of capacity. During reduction, the calculated volume expansion coincides with that expected from the equilibrium Li_xSn phases in lithia (blue squares). In particular, at the end of electrochemical reduction, a volume expansion of 258% is found, in

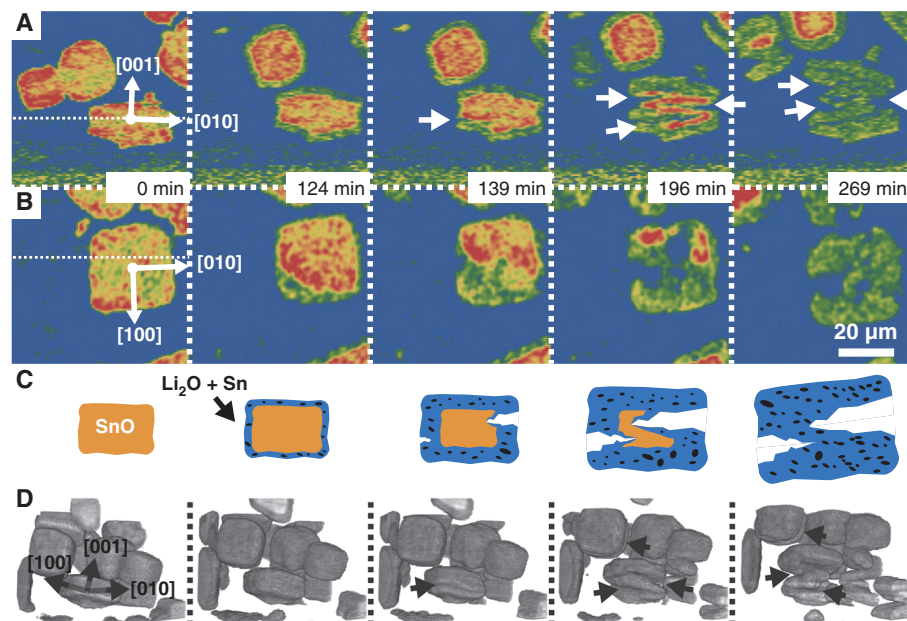


Fig. 3. Evolution of particle fracture. (A) Coronal and (B) transverse cross sections through a particle during electrochemical reduction. Horizontal dotted white lines in the cross sections at 0 min indicate cutting planes. Crystallographic directions are identified and indicated. White arrows point to crack locations. (C) Schematic of particle phase evolution and crack growth leading to zig-zag morphology. (D) 3D rendering of subvolume visualizing zig-zag morphology in multiple particles. Black arrows indicate fracture.

good agreement with theoretical predictions of 252% for Li₂O + Li_{4.4}Sn with respect to SnO.

As highlighted in movie S4, the volume expansion of the active particles drives expansion of the entire electrode, which can lead to mechanical breakdown of the polymeric binder and carbon black matrix (34). In Fig. 4C, snapshots of radiographs of a central section of the electrode are depicted during reduction and oxidation. The top edge of the electrode is indicated by a white line, and tracing its temporal evolution shows increasing electrode thickness during electrochemical reduction and decreasing thickness upon oxidation. To quantify the relation between the volume change of the active particles and thickness change in the electrodes, we calculate the volume fraction of active particles in the direction normal to the current collector (Fig. 4D) from slices 1 voxel high and 512 by 512 voxels in area. Plotting the maximum distance from the current collector at a specific volume fraction allows us to quantify the thickness change (Fig. 4E). We compare this to the temporal evolution of the average volume fraction of the active particles (Fig. 4F). The freshly prepared electrode measures 50 μ m, and the average active particle volume fraction is 0.24. During electrochemical reduction, the electrode expands to more than 120 μ m. At first, this electrode expansion occurs concomitantly with particle volume expansion, as evidenced by the roughly constant volume fraction. However, after the electrode thickness has doubled, even though the total electrode thickness continues to grow, the particles begin to occupy an increasingly large volume fraction of the elec-

trode, as demonstrated by the rise in active material volume fraction in the electrode. This behavior is indicative of particle volume expansion beyond what can be accommodated by the polymer binder and carbon black matrix. After oxidation, the electrode contracts to a thickness of 80 μ m, but the particle volume fraction decreases to 0.21, a value below the starting volume fraction, which implies that the polymer binder and carbon black matrix are permanently distorted in the first reduction step. This distortion of the conductive matrix, together with particle fracture, is known to electrically disconnect particles from the rest of the electrode, leading to capacity loss (1, 2).

To test whether our tomography data show evidence of electrically disconnected particles, we carefully inspect the attenuation coefficient histograms in fig. S3A. We observe broadening of the Li₂O + Li_xSn peak during oxidation, which we quantify in fig. S3B by plotting the full width at half maximum. Because the attenuation coefficient is related to the lithium content (x in Li₂O + Li_xSn), we attribute the spread in attenuation coefficient to a spread in lithium content across different particles and interpret this as a sign for electrical disconnection of individual particles from the rest of the electrode.

Our electrochemical data are consistent with this interpretation. For SnO, it is known that below 1.0 V, dealloying of Li_xSn is the only source of capacity. Above 1.5 V (shaded region in Fig. 4B), decomposition of Li₂O and reformation of Sn-O contacts, leading to unknown and possibly amorphous or disordered phases, have been reported

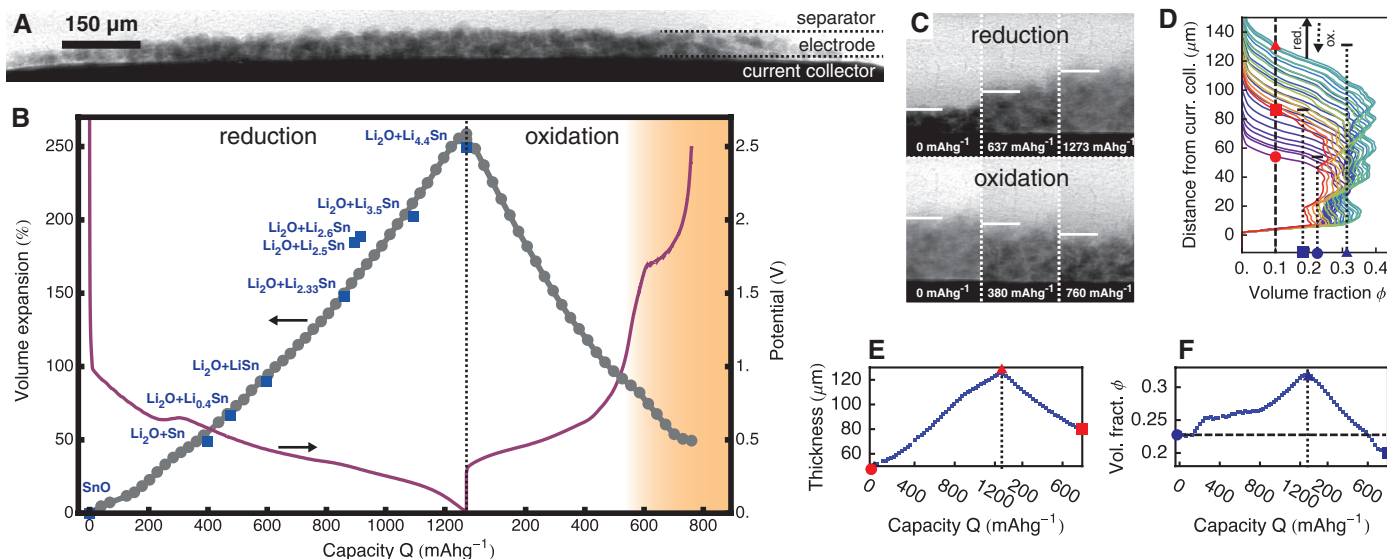


Fig. 4. Volume expansion. (A) X-ray radiograph of the full electrode highlights the accessible volume, which includes the entire electrode as well as the separator and current collector. (B) Quantitative volume expansion of the active materials (gray circles) in the entire electrode compared with equilibrium phases (blue squares) and cell voltage (purple line) during electrochemical reduction and oxidation. (C) Snapshots of radiographs from the electrode center show qualitative electrode expansion. White lines denote the top edge of the electrode. (D) Volume fraction of active particles in the electrode as a

function of the distance from the current collector (curr. coll.). Coloring indicates temporal evolution. Dotted lines indicate the average volume fraction and associated electrode thickness before reduction (circles), after reduction (triangles), and after oxidation (squares). Red symbols indicate thickness; blue symbols represent volume fraction. Following the distance from the current collector at a fixed volume fraction (vertical dashed line at 0.1 volume fraction) quantifies (E) the change in thickness of the electrode. (F) Average volume fraction of active particles in the electrode as a function of capacity.

(35). In our cell, the extracted capacity during oxidation below 1.5 V is $\sim 550 \text{ mA}\cdot\text{hour g}^{-1}$, $\sim 37\%$ below the theoretical value of $873 \text{ mA}\cdot\text{hour g}^{-1}$.

Finally, we test if the identified spread in lithium content identified in the tomography data can account for the electrochemically determined capacity loss. We estimate the state of charge, parameterized by the average lithium content $\hat{x}$, by numerically integrating over the $\text{Li}_2\text{O} + \text{Li}_x\text{Sn}$ attenuation coefficient peak, as discussed in the supplementary text. We find that the estimated lithium content is $\hat{x} = 1.9$ at a cell voltage of 1.5 V, which corresponds to a lost capacity of $\sim 43\%$, in good agreement with the 37% determined electrochemically. This finding demonstrates that SRXTM can track and quantify cause and effect of electrochemical and mechanical processes and enables a self-consistent description of battery degradation mechanisms.

The experiment presented here can be repeated for a number of anode and cathode materials to develop a comprehensive framework comprising all electrochemical and mechanical aspects of conversion and alloying materials. For example, this framework could resolve open questions associated with the lithiation of silicon, a potential anode material. Highly anisotropic lithiation has been observed in crystalline silicon micro- and nanostructures (18, 19), although lithium diffusion in silicon is expected to be isotropic (13). Current explanations include anisotropic volume expansion and unequal reaction kinetics for different crystal planes (13). Electrochemical amorphization and the sudden appearance of crystalline $\text{Li}_{15}\text{Si}_4$ from these amorphous phases

(36), stress-dependent potential of lithiated silicon (17), and a rate-independent polarization hysteresis (16) add to a complicated picture. However, the type of quantitative, 3D, and time-resolved images of particle lithiation introduced in this work will provide the experimental data necessary to comprehend the complex electrochemical and mechanical interactions in silicon and related materials. The development of chemistries and particle morphologies that hinder crack formation or growth, as well as expansion-tolerating composite electrode architectures, may enable better batteries.

References and Notes

- J. Cabana, L. Monconduit, D. Larcher, M. R. Palacin, *Adv. Mater.* **22**, E170–E192 (2010).
- W.-J. Zhang, *J. Power Sources* **196**, 877–885 (2011).
- Y. Oumellal, A. Rougier, G. A. Nazri, J.-M. Tarascon, L. Aymard, *Nat. Mater.* **7**, 916–921 (2008).
- A. S. Aricò, P. Bruce, B. Scrosati, J.-M. Tarascon, W. van Schalkwijk, *Nat. Mater.* **4**, 366–377 (2005).
- P. Poizot, S. Laruelle, S. Grugeon, L. Dupont, J.-M. Tarascon, *Nature* **407**, 496–499 (2000).
- M. N. Obrovac, L. Christensen, D. B. Le, J. R. Dahn, *J. Electrochem. Soc.* **154**, A849–A855 (2007).
- J. Y. Huang et al., *Science* **330**, 1515–1520 (2010).
- N. Balke et al., *Nano Lett.* **10**, 3420–3425 (2010).
- S.-C. Chao et al., *Electrochem. Commun.* **12**, 234–237 (2010).
- S.-C. Chao et al., *J. Phys. Chem. C* **115**, 22040–22047 (2011).
- S.-C. Chao et al., *J. Electrochem. Soc.* **158**, A1335–A1339 (2011).
- J. Wang, Y. C. Chen-Wiegart, J. Wang, *Chem. Commun.* **49**, 6480–6482 (2013).
- M. K. Y. Chan, C. Wolverton, J. P. Greeley, *J. Am. Chem. Soc.* **134**, 14362–14374 (2012).
- M. E. Stournara, P. R. Guduru, V. B. Shenoy, *J. Power Sources* **208**, 165–169 (2012).
- K. Zhao et al., *J. Electrochem. Soc.* **159**, A238–A243 (2012).
- V. A. Sethuraman, V. Srinivasan, J. Newman, *J. Electrochem. Soc.* **160**, A394–A403 (2013).
- V. A. Sethuraman, V. Srinivasan, A. F. Bower, P. R. Guduru, *J. Electrochem. Soc.* **157**, A1253–A1261 (2010).
- S. W. Lee, M. T. McDowell, L. A. Berla, W. D. Nix, Y. Cui, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 4080–4085 (2012).
- J. L. Goldman, B. R. Long, A. A. Gewirth, R. G. Nuzzo, *Adv. Funct. Mater.* **21**, 2412–2422 (2011).
- J. Chouvin et al., *J. Electroanal. Chem.* **494**, 136–146 (2000).
- T. Ohzuku, *J. Electrochem. Soc.* **144**, 3496–3500 (1997).
- J. O. Besenhard, M. Winter, J. Yang, W. Biberacher, *J. Power Sources* **54**, 228–231 (1995).
- P. Baudry, M. Armand, M. Gauthier, J. Masounave, *Solid State Ion.* **28–30**, 1567–1571 (1988).
- L. Y. Beaulieu, K. W. Eberman, R. L. Turner, L. J. Krause, J. R. Dahn, *Electrochem. Solid-State Lett.* **4**, A137–A140 (2001).
- M. Stampanoni et al., *Proc. SPIE* **6318**, 63180M (2006).
- F. Marone, M. Stampanoni, *J. Synchrotron Radiat.* **19**, 1029–1037 (2012).
- P. R. Shearing et al., *J. Electrochem. Soc.* **159**, A1023–A1027 (2012).
- M. Ebner, F. Geldmacher, F. Marone, M. Stampanoni, V. Wood, *Adv. Energy Mater.* **3**, 845–850 (2013).
- Materials and methods are available as supplementary materials on Science Online.
- I. A. Courtney, J. R. Dahn, *J. Electrochem. Soc.* **144**, 2045–2052 (1997).
- I. A. Courtney, J. R. Dahn, *J. Electrochem. Soc.* **144**, 2943–2948 (1997).
- I. A. Courtney, W. R. McKinnon, J. R. Dahn, *J. Electrochem. Soc.* **146**, 59–68 (1999).
- S. Wang et al., *Chem. Commun.* **2005**, 507–509 (2005).
- Z. Chen, L. Christensen, J. R. Dahn, *J. Electrochem. Soc.* **150**, A1073–A1078 (2003).
- I. A. Courtney, R. A. Dunlap, J. R. Dahn, *Electrochim. Acta* **45**, 51–58 (1999).

36. M. N. Obrovac, L. Christensen, *Electrochem. Solid-State Lett.* **7**, A93–A96 (2004).

Acknowledgments: The tomography experiments were performed on the Tomographic Microscopy and Coherent Radiology Experiments (TOMCAT) beamline at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland. We thank P. Modregger, L. Nowack, and M.-F. Lagadec for their support during the

beamtime; K. Kunze and W. Woodford for insightful discussion; D. Norris for access to SEM; and O. Waser and S. Pratsinis for access to XRD. We gratefully acknowledge material donations from TIMCAL and Arkema.

Supplementary Materials

www.sciencemag.org/content/342/6159/716/suppl/DC1
Materials and Methods

Supplementary Text

Figs. S1 to S3

Table S1

References (37, 38)

Movies S1 to S4

13 June 2013; accepted 3 October 2013

Published online 17 October 2013;
10.1126/science.1241882

The Role of Surface Oxygen in the Growth of Large Single-Crystal Graphene on Copper

Yufeng Hao,¹ M. S. Bharathi,² Lei Wang,³ Yuanyue Liu,⁴ Hua Chen,⁵ Shu Nie,⁶ Xiaohan Wang,¹ Harry Chou,¹ Cheng Tan,¹ Babak Fallahazad,⁷ H. Ramanarayan,² Carl W. Magnuson,¹ Emanuel Tutuc,⁷ Boris I. Yakobson,⁴ Kevin F. McCarty,⁶ Yong-Wei Zhang,² Philip Kim,⁸ James Hone,³ Luigi Colombo,^{9*} Rodney S. Ruoff^{1,*}

The growth of high-quality single crystals of graphene by chemical vapor deposition on copper (Cu) has not always achieved control over domain size and morphology, and the results vary from lab to lab under presumably similar growth conditions. We discovered that oxygen (O) on the Cu surface substantially decreased the graphene nucleation density by passivating Cu surface active sites. Control of surface O enabled repeatable growth of centimeter-scale single-crystal graphene domains. Oxygen also accelerated graphene domain growth and shifted the growth kinetics from edge-attachment-limited to diffusion-limited. Correspondingly, the compact graphene domain shapes became dendritic. The electrical quality of the graphene films was equivalent to that of mechanically exfoliated graphene, in spite of being grown in the presence of O.

Control of the nucleation and growth of graphene during the chemical vapor deposition (CVD) process is important to achieve large, high-quality single crystals (1–5). Much attention has been paid to the process details, with emphasis on the parameters of carbon (C) precursors, hydrogen (H), copper (Cu), temperature, and pressure (6). As such, tuning the C:H ratio (6), changing the hydrocarbon and H₂ gas pressures (7), and smoothing the Cu surface before growth (8, 9) have been used to grow graphene with desirable quality. However, the wide variation in domain size, shape, and film quality from lab to lab suggests that crucial growth parameters still remain unknown or uncontrolled. We show that oxygen (O) on the Cu surface not only suppresses graphene nucleation, fostering growth of ultralarge single-crystal graphene do-

main, but also lowers the C species edge attachment barrier and shifts the graphene domain shapes from compact to dendritic (10). First-principles calculations and phase field simulations provided a deeper insight into the proposed growth mechanism and reproduced the observed domain shapes.

Oxygen impurities were found to exist at different concentrations across commercially available Cu foils. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) depth profile results [fig. S2B, see supplementary materials (11)] show that, for two different types of Cu foils, the O concentrations are $\sim 10^{-2}$ and $\sim 10^{-6}$ atomic % (the

latter approaching the detection limit), hereafter referred to as “oxygen-rich Cu” (OR-Cu) and “oxygen-free Cu” (OF-Cu), respectively. When the two types of Cu were used to grow graphene under the same conditions in low-pressure CVD, the domain density for OR-Cu was $\sim 0.9 \text{ mm}^{-2}$, more than three orders of magnitude lower than that for OF-Cu, which was about $2 \times 10^3 \text{ mm}^{-2}$ (Fig. 1, A and B). We also observed that graphene domains on OR-Cu always exhibited dendritic growth fronts, i.e., multibranch and rough domain edges (Fig. 1A, inset), whereas graphene domains on OF-Cu were compact with sharp edges (Fig. 1B, inset). When we exposed OF-Cu to O₂ [partial pressure of O₂ (P_{O_2}) = 1×10^{-3} torr; the substrates are defined as “OF-Cu (O)” hereafter] for 1 min before introducing methane (CH₄, P_{CH_4} = 1×10^{-3} torr), the resulting graphene growth yielded a low density of nuclei, $\sim 6 \text{ mm}^{-2}$, and dendritic growth fronts (Fig. 1C) similar to those on OR-Cu. In addition, TOF-SIMS results (fig. S2A) showed the presence of surface O after O₂ exposure and annealing in H₂ (P_{H_2} = 0.1 torr). Because graphene growth on Cu is a surface-mediated process (12), it is reasonable that surface O species, either segregated out of the Cu bulk or adsorbed from O₂ exposure, participate in surface reactions and are thus responsible for the domain growth characteristics. Both experimental (13, 14) and theoretical studies (15) have established that metal surface imperfections, such as step edges, defects, impurities, etc., can be active sites for graphene nucleation because of higher *d*-band centers at these lower-coordination sites, which lead to strong binding to adsorbates (16, 17). For the same reasons, these active sites are also sinks for O. Thus, surface O on the Cu, regardless

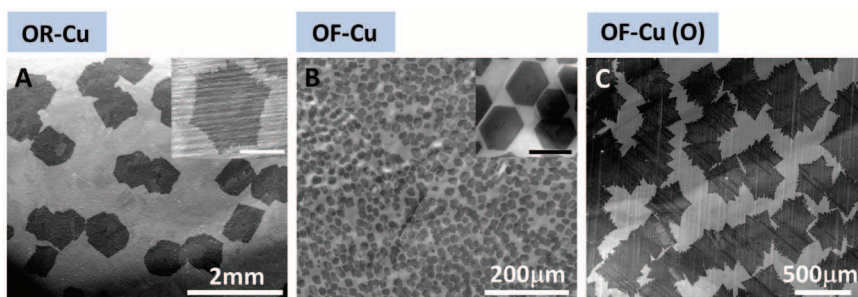


Fig. 1. The effect of O on graphene nucleation density and domain shapes on Cu. Scanning electron microscope (SEM) images of graphene domains grown on (A) OR-Cu, (B) OF-Cu, and (C) OF-Cu (O), respectively. In all cases, the P_{CH_4} = 1×10^{-3} torr, and P_{H_2} = 0.1 torr, and the growth time was 150 min for (A) and (C) and 50 min for (B). The insets in (A) and (B) are the high-magnification SEM images in each case. The scale bar is 500 μm in the inset of (A) and 20 μm in the inset of (B).

¹Department of Mechanical Engineering and the Materials Science and Engineering Program, The University of Texas at Austin, Austin, TX 78712, USA. ²Institute of High Performance Computing, A*STAR, 138632, Singapore. ³Department of Mechanical Engineering, Columbia University, New York, NY 10027, USA. ⁴Department of Mechanical Engineering and Materials Science, and Department of Chemistry, Rice University, Houston, TX 77005, USA. ⁵Department of Physics, The University of Texas at Austin, Austin, TX 78712, USA. ⁶Sandia National Laboratories, Livermore, CA 94550, USA. ⁷Microelectronics Research Center, The University of Texas at Austin, Austin, TX 78758, USA. ⁸Department of Physics, Columbia University, New York, NY 10027, USA. ⁹Texas Instruments, Dallas, TX 75243, USA.

*Corresponding author. E-mail: r.ruoff@mail.utexas.edu (R.S.R); colombo@ti.com (L.C.)

of its source, effectively passivated the surface active sites where hydrocarbon accumulation would otherwise have taken place.

In order to further suppress graphene nucleation on Cu, we exposed the Cu substrates to

varying amounts of O_2 ($P_{O_2} = 1 \times 10^{-3}$ torr) by simply increasing the exposure time up to 5 min before introducing CH_4 . Typically, 2 min of O_2 exposure on OR-Cu can decrease the nucleation density to $\sim 0.03 \text{ mm}^{-2}$ (Fig. 2A). With longer O_2

exposure (5 min), the graphene nucleation density was as low as $\sim 0.01 \text{ mm}^{-2}$, and individual domains grew to a diameter larger than 1 cm after a 12-hour growth period at $P_{CH_4} = 1 \times 10^{-3}$ torr (Fig. 2B). The same experiments with OF-Cu

Fig. 2. Size, structure, and electrical transport properties of large graphene domains grown on Cu exposed to O_2 . (A) SEM image of low-density graphene domains on OR-Cu exposed to O_2 . (B) Optical image of centimeter-scale graphene domains on OR-Cu exposed to O_2 . The sample was prepared by heating in air at 180°C for 30 min to oxidize bare Cu (orange) and visualize graphene domains (pale area), as reported by Wang *et al.* (9). (C) Superimposed SEM and EBSD images of a graphene domain grown across Cu multigrains. (D) The graphene nucleation density as a function of O_2 exposure time. (E) Plots of resistivity and conductivity as a function of gate voltage at 1.7 K. (F) Longitudinal resistivity, R_{xx} , measured on left axis (black) and Hall resistance, R_{xy} , on right axis (red) as a function of V_g .

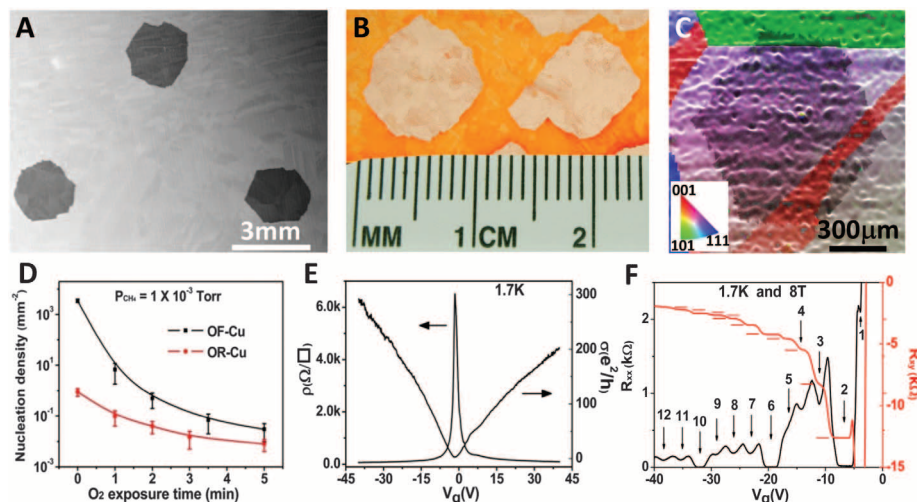
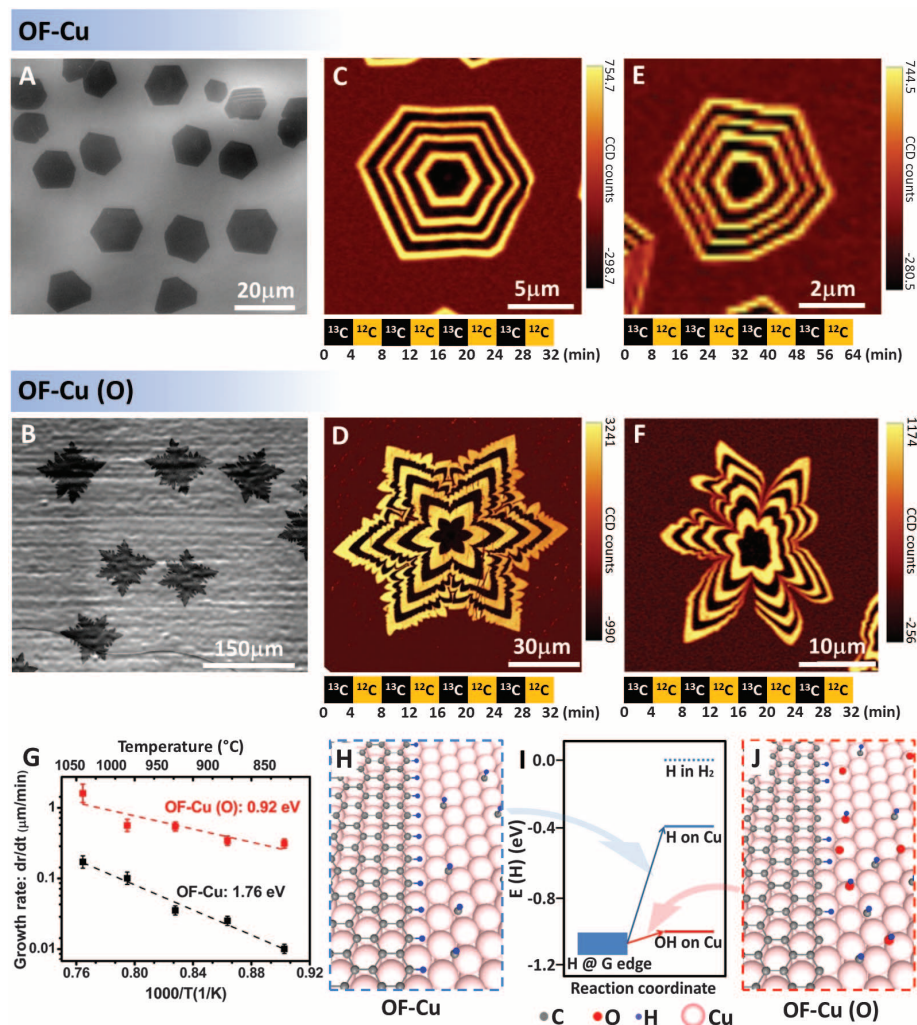


Fig. 3. The effect of O on graphene growth kinetics. SEM images of graphene domains grown on (A) OF-Cu and (B) OF-Cu (O). Isotope-labeled Raman maps of the 2D (G') band intensities on Si substrates for growth at (C and D) 1035°C and (E and F) 885°C . The isotope switching intervals are indicated below each image. (G) Logarithmic plots of graphene domain growth rate $d\bar{r}/dt$ versus $1/T$. The error bars are from calculations of different domains for each case, and the activation energy E_a is extracted from the slope of the linear fit. (H and I) Atomic-scale schematics of graphene edge growth on Cu with and without the assistance of O, respectively. (J) DFT calculated energies of different configurations of H attachment, in reference to H in H_2 . The energy spread of H at the graphene edge is due to the computational uncertainty resulting from the lattice mismatch between graphene and Cu.



gave rise to a similar trend; i.e., the nucleation density decreased with increasing O_2 exposure time (Fig. 2D). Oxygen exposure provided a convenient tuning parameter for suppressing graphene nucleation and growing large domains. We used electron backscatter diffraction (EBSD) to map the crystalline orientations of Cu grains under a graphene domain. The results show that large graphene domains normally grow across several Cu grains, which usually have a grain size smaller than a few millimeters even after annealing (Fig. 2C). Low-energy electron diffraction (LEED) patterns (fig. S8) taken at different positions on the same domain show that, even though the underlying Cu is multigrain, all of the graphene diffraction patterns are aligned with each other, indicating a single-crystal graphene domain. LEED measurements were also performed on other randomly selected large graphene domains with varying shapes (such as in Fig. 2B) on multigrain Cu and confirmed that the domains are single crystals. These observations suggest that single-crystal Cu substrates are unnecessary to grow large single-crystal graphene films. In addition, we found that high growth temperatures and low P_{CH_4} facilitated single-crystal graphene growth. Raman spectra of the domains transferred onto silicon (Si) substrates confirmed that they are single-layer with no detectable defect-related D band (fig. S5).

Electrical- and magneto-transport measurements were then performed on Si (fig. S11) and hexagonal boron nitride (h-BN) substrates. Resistivity as a function of the back gate voltage of graphene films on h-BN (Fig. 2E) shows narrow and symmetric Dirac peaks with the charge neutrality point $V_g = -1.0$ V. The carrier mobility measured for three different samples ranged from 40,000 to 65,000 $cm^2 V^{-1} s^{-1}$ at 1.7 K and from 15,000 to 30,000 $cm^2 V^{-1} s^{-1}$ at room temperature when a carrier density-independent fitting method was used (18). Magneto-transport measurements show quantum Hall states at all integer filling factors from 1 to 12 at a magnetic field of 8 T (Fig. 2F), indicating that the fourfold degeneracy of the Landau levels is lifted. The onset of Shubnikov-de Haas oscillations was also observed at fields below 500 mT (fig. S12). These features suggest that the electrical quality of large graphene domains, despite having been grown with O on Cu, is among the best reported for CVD graphene (19) and comparable to that of micro-mechanically exfoliated graphene (20).

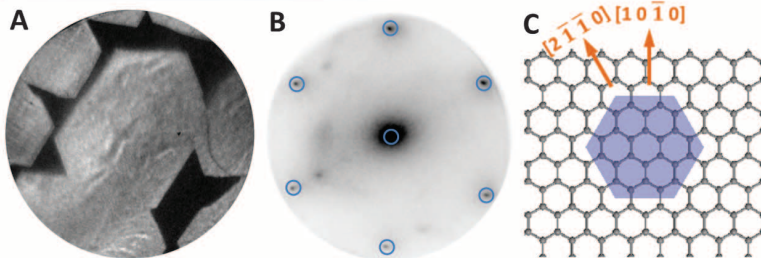
In addition to decreasing graphene nucleation density, O affected graphene growth kinetics. Figure 3A shows that graphene domains on OF-Cu were compact with a domain size of $\sim 15 \mu m$ after 32 min of growth at 1035°C and $P_{CH_4} = 2 \times 10^{-3}$ torr. However, when graphene was grown on OF-Cu (O) ($P_{O_2} = 1 \times 10^{-3}$ torr, 30 s of exposure) under the same conditions and for the same growth time, the domain size increased to $\sim 100 \mu m$ (Fig. 3B). This acceleration of graphene domain growth by surface O may seem counterintuitive, because O has been associated with C species oxidation

and graphene etching (21). Oxygen also decreased the graphene film coverage on the Cu substrates by 5 to 10 times after 32 min of growth (11). This decrease resulted from the nucleation density on OF-Cu (O) being more than two orders of magnitude lower despite the higher individual domain growth rate (11).

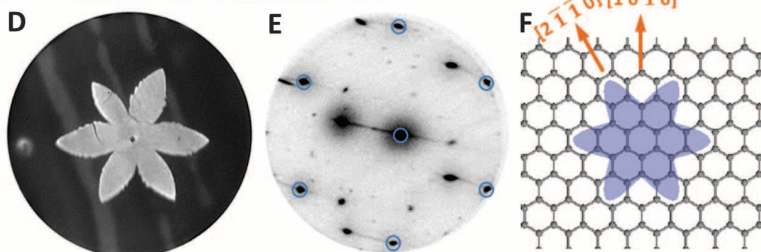
We visualized the time evolution of domain growth at different growth temperatures with C isotope labeling and Raman mapping (Fig. 3, C to F, and fig. S7). Over a wide temperature range,

the domains on OF-Cu remained compact hexagons as they grew, whereas on OF-Cu (O), the domains were always multibranched and dendritic. The consistent domain shapes suggest that the kinetics do not change throughout the growth. Also, the radial growth rates of individual domains were nearly constant along a given orientation, as measured by the widths of the isotopically labeled bands. We plotted the growth rate as a function of temperature (Fig. 3G). According to the Arrhenius equation $d\bar{r}/dt \propto \exp(-E_a/k_B T)$,

OF-Cu



OF-Cu (O)



OF-Cu (O)

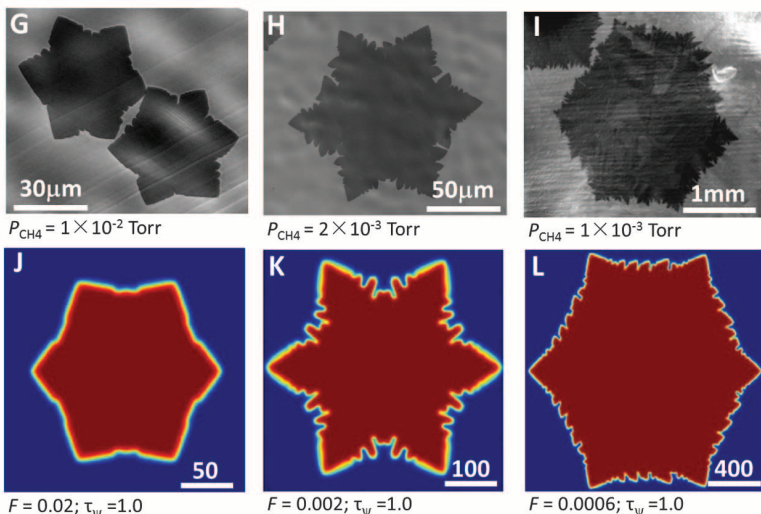


Fig. 4. The effect of O on graphene domain shapes on Cu. (A, B, D, and E) Low-energy electron microscopy images and corresponding LEED patterns (blue circles) of graphene domains on OF-Cu and OF-Cu (O), respectively. The extra LEED spots came from the faceted Cu. The viewing fields in (A) and (D) are 20 and 60 μm , respectively. (C and F) Schematics of growth directions of the two types of graphene domains. (G to I) SEM images of graphene domains grown on OF-Cu (O) as a function of P_{CH_4} . (J to L) Phase field simulation results as a function of characteristic attachment time and C flux. The scale bars in (J) to (L) refer to simulation units, corresponding to length.

where $\bar{r}$ is the average radius (11), E_a is the growth activation energy, k_B is the Boltzmann constant, and T is temperature. The value of E_a was 1.76 eV for graphene on OF-Cu and 0.92 eV on OF-Cu (O), indicating that, in the dynamic growth process, the barrier of the rate-limiting step is reduced.

For hydrocarbon (e.g., CH_4) conversion to graphene on Cu during CVD growth, the following elementary steps are expected (3, 22, 23): (i) CH_4 adsorption on Cu surfaces; (ii) CH_4 (partial-) dehydrogenation, resulting in C species such as CH_x ($x = 0$ to 3); (iii) surface diffusion of C species; and (iv) C species attachment to the graphene domain edge and incorporation into the graphene lattice. The dehydrogenation of CH_4 on Cu is endothermic (energetically unfavorable), and the diffusing C species on Cu are mainly CH_x ($0 < x < 4$), rather than atomic C (22). Density functional theory (DFT) calculations revealed that the H-terminated graphene edge on Cu is more energetically favorable than the bare graphene edge on Cu (Fig. 3, H and I). Thus, C species edge attachment and lattice incorporation require dehydrogenation [e.g., $\text{CH}_x \xrightarrow{\Delta} \text{CH}_{x-1} + \text{H}$ ($x = 4, 3, 2, 1$)], which is considered rate-limiting (3, 22, 23). In contrast, theoretical studies have shown that preadsorbed O on the Cu surface can enhance the dissociation of hydrocarbons (Fig. 3J) through the reaction $\text{CH}_x + \text{O} \xrightarrow{\Delta} \text{CH}_{x-1} + \text{OH}$ ($x = 4, 3, 2, 1$) (24, 25). Our DFT calculations have shown that the energy of H in the form of an OH group on Cu is lower than that of H on Cu by 0.6 eV/H (Fig. 3I), pointing to a lower activation energy of edge dehydrogenation according to the Bell-Evans-Polanyi principle (26). Thus, experimental data from the isotope-labeled growth and the atomic-scale calculations reveal that O helps reduce the edge attachment barrier, facilitates C incorporation, and accelerates graphene growth. In the process, graphene growth proceeded by continuous C edge attachment and lattice incorporation, whereas surface O species were consumed and then desorbed. This scenario does not necessarily conflict with the passivation effect of O on graphene nucleation. The latter is governed by different kinetic processes (23) that typically require a much higher supersaturation density of C species than that during growth, in which dehydrogenation may not be the critical step.

The above model is supported by the change of graphene domain morphology with O_2 exposure. Here, we focus on the domains on Cu(111). Figure 4A shows the typical shape of graphene domains formed on OF-Cu: compact hexagons with sharp edges, as obtained from the kinetic Wulff construction (27), which is expected as a result of edge-attachment-limited growth. In contrast, graphene domains grown on OF-Cu (O) became multibranched or dendritic (Fig. 4D), which is typical of diffusion (mass transport)-limited growth (3, 28). The morphology change indicates that, with the introduction of O, C attachment at domain edges is no longer rate-limiting, and domain growth is instead governed by C diffusion

or equivalently C flux, in agreement with our observations and analysis. The corresponding LEED patterns (Fig. 4, B and E) show only one set of hexagonal diffraction patterns, indicating that both domains are single crystals. Figure 4, C and F, sketch the relationship between the domain shapes and the graphene lattice. Both domains exhibit fast growth in the $[2\bar{1}\bar{1}0]$ direction and slow growth in the $[10\bar{1}0]$ direction, consistent with previous work, in which hexagonal domains have been reported to have zigzag-terminated edges (29). Oxygen affected the domain shapes by modifying the growth kinetics but preserved the sixfold crystallographic symmetry. We further found that graphene domains on OF-Cu maintain a hexagonal shape when P_{CH_4} ranges from 1×10^{-3} to 5×10^{-2} torr (fig. S9). However, on OF-Cu (O), different domain shapes appear as a function of P_{CH_4} (Fig. 4, G to I). The sensitive dependence of domain morphology on C concentration again suggests that the growth kinetics has been brought into the diffusion-limited regime by O.

To test the proposed growth mechanisms, a phase field model was developed to examine the domain shape evolution (11). Two key parameters, namely the characteristic attachment time of C species (τ_ψ) and C flux (F , reflecting the P_{CH_4}) are varied to simulate the experimental conditions. The attachment time is closely related to the edge attachment barrier: The higher the energy barrier, the longer the characteristic attachment time. The symmetry of the graphene domains is dictated by the sixfold graphene edge energy, in agreement with experimental observations. The simulated domain shapes are shown in Fig. 4, J to L, and fig. S9. When τ_ψ is long, the domain shape is hexagonal even if F is changed within a large range; whereas when τ_ψ decreases, the hexagonal domain turns into a six-branched domain at the same F . Furthermore, at the low τ_ψ value, F becomes the dominant parameter and can tune the domain shapes from six-branched to dendritic. Thus, lowering the attachment barrier changes the domain shape in the same manner as O_2 exposure in experiments. Thus, a rich variety of graphene domain morphologies (30, 31) can be explained and reproduced when the O effect is considered in the growth kinetics.

References and Notes

- X. Li et al., *Science* **324**, 1312–1314 (2009).
- S. Bae et al., *Nat. Nanotechnol.* **5**, 574–578 (2010).
- N. C. Bartelt, K. F. McCarty, *MRS Bull.* **37**, 1158–1165 (2012).
- A. W. Tsen et al., *Science* **336**, 1143–1146 (2012).
- P. M. Ajayan, B. I. Yakobson, *Nat. Mater.* **10**, 415–417 (2011).
- S. Bhaviripudi, X. Jia, M. S. Dresselhaus, J. Kong, *Nano Lett.* **10**, 4128–4133 (2010).
- I. Vlasiouk et al., *ACS Nano* **5**, 6069–6076 (2011).
- Z. Yan et al., *ACS Nano* **6**, 9110–9117 (2012).
- H. Wang et al., *J. Am. Chem. Soc.* **134**, 3627–3630 (2012).
- Z. Zhang, M. G. Lagally, *Science* **276**, 377–383 (1997).
- Materials and methods are available as supplementary materials on Science Online.
- X. Li, W. Cai, L. Colombo, R. S. Ruoff, *Nano Lett.* **9**, 4268–4272 (2009).
- S. Nie, J. M. Wofford, N. C. Bartelt, O. D. Dubon, K. F. McCarty, *Phys. Rev. B* **84**, 155425 (2011).
- G. H. Han et al., *Nano Lett.* **11**, 4144–4148 (2011).
- J. Gao, J. Yip, J. Zhao, B. I. Yakobson, F. Ding, *J. Am. Chem. Soc.* **133**, 5009–5015 (2011).
- H. Chen, W. Zhu, Z. Zhang, *Phys. Rev. Lett.* **104**, 186101 (2010).
- B. Hammer, J. K. Nørskov, *Adv. Catal.* **45**, 71–129 (2000).
- E. H. Hwang, S. Adam, S. D. Sarma, *Phys. Rev. Lett.* **98**, 186806 (2007).
- N. Petrone et al., *Nano Lett.* **12**, 2751–2756 (2012).
- C. R. Dean et al., *Nat. Nanotechnol.* **5**, 722–726 (2010).
- E. Starodub, N. C. Bartelt, K. F. McCarty, *J. Phys. Chem. C* **114**, 5134–5140 (2010).
- W. Zhang, P. Wu, Z. Li, J. Yang, *J. Phys. Chem. C* **115**, 17782–17787 (2011).
- H. Kim et al., *ACS Nano* **6**, 3614–3623 (2012).
- I. Alstrup, I. Chorkendorff, S. Ullmann, *Surf. Sci.* **264**, 95–102 (1992).
- B. Xing, X. Y. Pang, G. C. Wang, *J. Catal.* **282**, 74–82 (2011).
- R. A. van Santen, M. Neurock, S. G. Shetty, *Chem. Rev.* **110**, 2005–2048 (2010).
- V. I. Artyukhov, Y. Liu, B. I. Yakobson, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 15136–15140 (2012).
- T. A. Witten Jr., L. M. Sander, *Phys. Rev. Lett.* **47**, 1400–1403 (1981).
- Q. Yu et al., *Nat. Mater.* **10**, 443–449 (2011).
- B. Wu et al., *NPG Asia Mater.* **5**, e36 (2013).
- A. T. Murdock et al., *ACS Nano* **7**, 1351–1359 (2013).

Acknowledgments: We thank V. B. Shenoy (University of Pennsylvania), Zhenyu Zhang [University of Science and Technology of China (USTC)], Zhenyu Li (USTC), N. C. Bartelt (Sandia Laboratories), P. Sutter (Brookhaven Laboratory), Gui-Chang Wang (Nankai University), Cheng Gong (University of Texas–Dallas), Zhen Yan (Texas A&M University), and C. R. Dean (City College of New York) for valuable discussions. We thank K. Watanabe and T. Taniguchi for providing h-BN substrates. This work acknowledges support from the W. M. Keck Foundation, the Office of Naval Research (ONR), and the South West Academy of Nanoelectronics of the Nanoelectronics Research Initiative. Work at Columbia University was supported by the Center for Re-Defining Photovoltaic Efficiency through Molecular-Scale Control, an Energy Frontier Research Center funded by the U.S. Department of Energy (DOE), Office of Science, Office of Basic Energy Sciences under award DE-SC0001085, National Science Foundation (NSF) grant DMR-1124894, and ONR grant N000141310662. Work at the Institute of High Performance Computing was supported by the Agency for Science, Technology And Research (A*STAR), Singapore. Work at Sandia was supported by the Office of Basic Energy Sciences, Division of Materials and Engineering Sciences, U.S. DOE, under contract no. DE-AC04-94AL85000. Work at Rice University was supported by the ONR and NSF's Chemical, Bioengineering, Environmental, and Transport Systems Division. The first-principles computations were performed on Kraken at the National Institute for Computational Sciences (NSF grant OCI-1053575), Hopper at the National Energy Research Scientific Computing Center (DOE grant DE-AC02-05CH11231), and DaVinci at Rice University (NSF grant OCI-0959097). H.C. acknowledges support from NSF grant DMR-1122603. A relevant patent application is in process.

Supplementary Materials

www.sciencemag.org/content/342/6159/720/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S12
References (32–45)

29 July 2013; accepted 1 October 2013
10.1126/science.1243879

Asymmetric Distribution of Lunar Impact Basins Caused by Variations in Target Properties

Katarina Miljković,^{1*} Mark A. Wieczorek,¹ Gareth S. Collins,² Matthieu Laneuville,¹ Gregory A. Neumann,³ H. Jay Melosh,⁴ Sean C. Solomon,^{5,6} Roger J. Phillips,⁷ David E. Smith,⁸ Maria T. Zuber⁸

Maps of crustal thickness derived from NASA's Gravity Recovery and Interior Laboratory (GRAIL) mission revealed more large impact basins on the nearside hemisphere of the Moon than on its farside. The enrichment in heat-producing elements and prolonged volcanic activity on the lunar nearside hemisphere indicate that the temperature of the nearside crust and upper mantle was hotter than that of the farside at the time of basin formation. Using the iSALE-2D hydrocode to model impact basin formation, we found that impacts on the hotter nearside would have formed basins with up to twice the diameter of similar impacts on the cooler farside hemisphere. The size distribution of lunar impact basins is thus not representative of the earliest inner solar system impact bombardment.

Progress in understanding impact basins on the Moon has been hampered by the simple fact that there is a lack of consensus on the size of the largest basins (1–3). From an impact physics perspective, the most relevant metric for the size of a basin is the diameter of its transient cavity, but as its name implies, this structure is short-lived and its diameter is not easily estimated from surface measurements (4). Most impact basins on the nearside hemisphere of the Moon have been filled by lava flows, hiding important morphological clues that could be used for determining the size of the transient cavity. Other impact basins have multiple rings, and it is unclear which of these, if any, most closely approximates the transient cavity. Because the impact process excavates large quantities of crustal material and uplifts mantle material beneath the basin center, an alternative metric for the size of a basin is the diameter of the region of crustal thinning (5–7). High-resolution gravity data obtained from NASA's Gravity Recovery and Interior Laboratory (GRAIL) mission (8) have provided global maps of crustal thickness on the Moon (9) that allow for an unambiguous determination of the region of crustal thinning for all impact basins with diameters greater than 200 km.

GRAIL gravity data show that lateral variations in the Moon's crustal thickness are domi-

nated by impact basins ranging in diameter from ~200 to 2000 km (9). Approximately half of those basins formed in the Imbrian and Nectarian periods, from ~3.7 to perhaps 4.2 billion years ago (Ga) (10, 11) (table S1). The sole exception is the South Pole–Aitken basin, which is the oldest and largest impact structure on the Moon, and which we do not consider further on the grounds that it likely formed during a much earlier epoch than the other basins for which variations in crustal thickness have been preserved. We quantify the size of lunar impact basins by the diameter D of the region of crustal thinning (1). There are 12 basins on each hemisphere with diameters greater than 200 km and crust thinned to a few kilometers, as resolved by GRAIL (Fig. 1). Although the to-

tal number of basins is equal on the two hemispheres, their size distribution is highly asymmetric (Fig. 2). Whereas there are eight basins on the nearside hemisphere with diameters greater than 320 km, only one of this size is found on the farside, and this basin (Orientale, 94°W, 20°S) straddles the western limb of the Moon. Simulations of the Moon's impact bombardment by near-Earth asteroids show that the difference in cratering rate between the nearside and farside hemispheres should be less than 1% (12) for a large range of impact conditions. With a uniform cratering rate, there is less than 2% probability that eight basins with diameters greater than 320 km would form on the nearside and only one such basin on the farside (fig. S1).

The Moon shows major geological differences between the nearside and farside hemispheres. The nearside is dominated by the compositionally unique Procellarum KREEP Terrane (PKT), which is highly enriched in heat-producing and other incompatible elements [potassium, rare-earth elements, and phosphorus (KREEP)] that likely formed during the late stages of magma-ocean crystallization (13, 14) (Fig. 1). More than 99% by area of the Moon's exposed basaltic lavas erupted on the lunar nearside; this concentration has been attributed to higher than average nearside mantle temperatures, at least in part the result of the high concentration of heat-producing elements in the nearside crust and upper mantle (15). The evidence for viscous relaxation of topographic relief of nearside basins (16, 17) and the presence of mare basalts extending beyond the confines of the surface area of thorium enrichment (which defines the PKT) suggest that higher than average subsurface temperatures surrounded

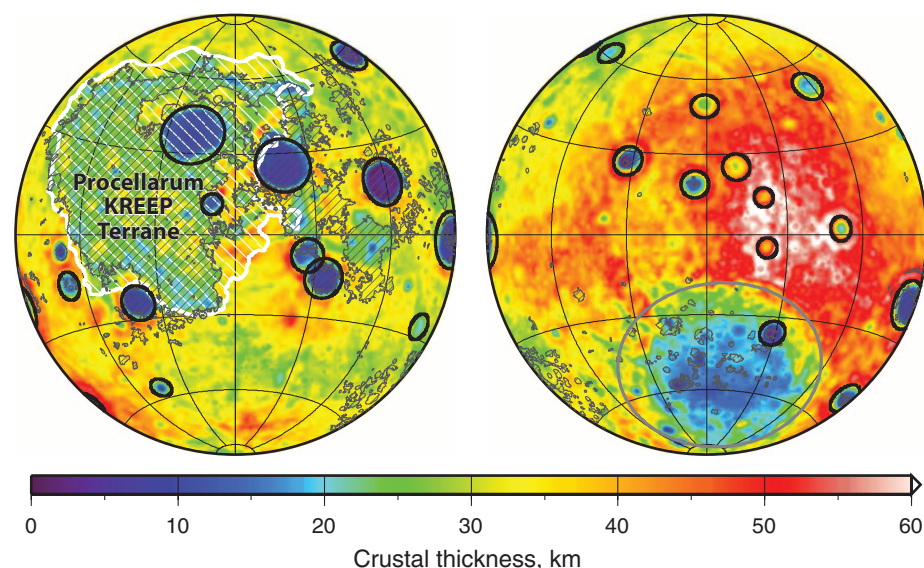


Fig. 1. Global map of crustal thickness on the Moon derived from GRAIL gravity data. Shown is the Procellarum KREEP Terrane (PKT; white cross-hatching), defined by the 4-ppm contour of thorium (32), and the distribution of mare basalt (black cross ruling). Excluding the South Pole–Aitken basin (gray circle), there are 12 impact basins with diameters of crustal thinning greater than 200 km (black circles) on each hemisphere. This image is presented in two hemispherical Lambert azimuthal equal-area projections centered over the nearside (left) and farside (right) hemispheres.

¹Institut de Physique du Globe de Paris, Sorbonne Paris Cité, Université Paris Diderot, Case 7011, Lamarck A, 5, 35 rue Hélène Brion, 75205 Paris cedex 13, France. ²Department of Earth Sciences and Engineering, Imperial College London, South Kensington Campus, London SW7 2AZ, UK. ³Solar System Exploration Division, NASA Goddard Space Flight Center, Greenbelt, MD 20771, USA. ⁴Department of Earth, Atmospheric, and Planetary Sciences, Purdue University, West Lafayette, IN 47907, USA. ⁵Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, DC 20015, USA. ⁶Lamont-Doherty Earth Observatory, Columbia University, Palisades, NY 10964, USA. ⁷Planetary Science Directorate, Southwest Research Institute, Boulder, CO 80302, USA. ⁸Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*Corresponding author. E-mail: miljkovic@ipgg.fr

the PKT for a considerable interval of lunar history. Several models have been proposed to account for the hemispheric differences in volcanic activity and heat-producing elements, all of which predict hemispheric differences in crustal and upper mantle temperatures (15, 18–20). We propose that hemispheric differences in subsurface temperature and, to a lesser extent, crustal thickness (9) are the cause of the asymmetric distribution of large impact basins. We tested this hypothesis using numerical simulations of impact basin formation.

To investigate the consequences of impact basin formation on the two lunar hemispheres, we used the iSALE-2D shock-physics hydrocode (21–23). Vertical impacts onto the lunar surface were modeled with impact speeds of 10 and 17 km s⁻¹. From available material models and following previous work (24, 25), we used basalt and dunite to represent the lunar crust and mantle, respectively, and dunite to represent the impactor (table S2). The pre-impact crustal thicknesses for the nearside and farside were set to 30 and 60 km, respectively. Representative subsurface temperature profiles beneath the nearside PKT and the farside hemisphere during the basin-forming epoch (4 Ga) were obtained from a three-dimensional thermochemical convection code (26) that included the asymmetric heat source distribution associated with the PKT (20) and provided results similar to those of previous asymmetric models (15)

(fig. S2). For a given impact velocity and impactor diameter, six impact simulations were performed, each with different temperature profiles for each hemisphere (tables S3 and S4).

Our simulations show that lunar impact basins form via the growth of a deep, bowl-shaped transient cavity that is gravitationally unstable and that collapses by a combination of uplift of the crater floor and inward collapse of the crater rim (7, 25). The crustal structure is modified in several ways during this process (fig. S4). During the formation of the transient crater, crustal material is ejected from inside the transient crater rim and deposited outside the transient crater; this process thins the crust inside the crater rim and thickens it outside (fig. S4). Because the size of the transient crater is limited by the impact energy available to displace the excavated mass in the ambient gravity field, the diameter of crustal thinning at this intermediate stage depends primarily on the impactor mass and speed and only weakly on the ambient temperature or crustal thickness. However, the subsequent collapse of the transient crater, and the consequent modifications in crustal structure, depend sensitively on the shear strength of the crust and upper mantle, which is a strong function of temperature. On the cooler and stronger farside, as the mantle beneath the crater floor is uplifted, crust beneath the transient crater rim collapses inward, forming a collar of crust around the mantle uplift and resulting in a

diameter of thinned crust that is smaller than the transient crater diameter. In contrast, the collapse of the transient crater on the warmer and weaker nearside is more extensive: The mantle below the crater floor is uplifted farther and over a much broader region, which prevents the thickened crust surrounding the transient crater from collapsing back into the crater. As a result, the diameter of crustal thinning is substantially larger on the hot nearside than on the cold farside (Fig. 3).

The diameter of crustal thinning for a lunar basin formed in the nearside thin crust is plotted in Fig. 4 as a function of the diameter that would occur for the same impact in the farside thick crust. The crustal thinning diameter does not differ markedly between the two hemispheres when the same temperature profile is used. Nevertheless, as demonstrated by crustal thickness profiles in Fig. 3, the ambient crustal thickness does have an influence on the character of the final crustal thickness profile. In contrast, for the same impact conditions, the crustal thinning diameter is greatly affected by temperature profile. Despite forming a nearly identical transient cavity, nearside basins formed in a hot target can have diameters of thinned crust with up to twice the diameter of

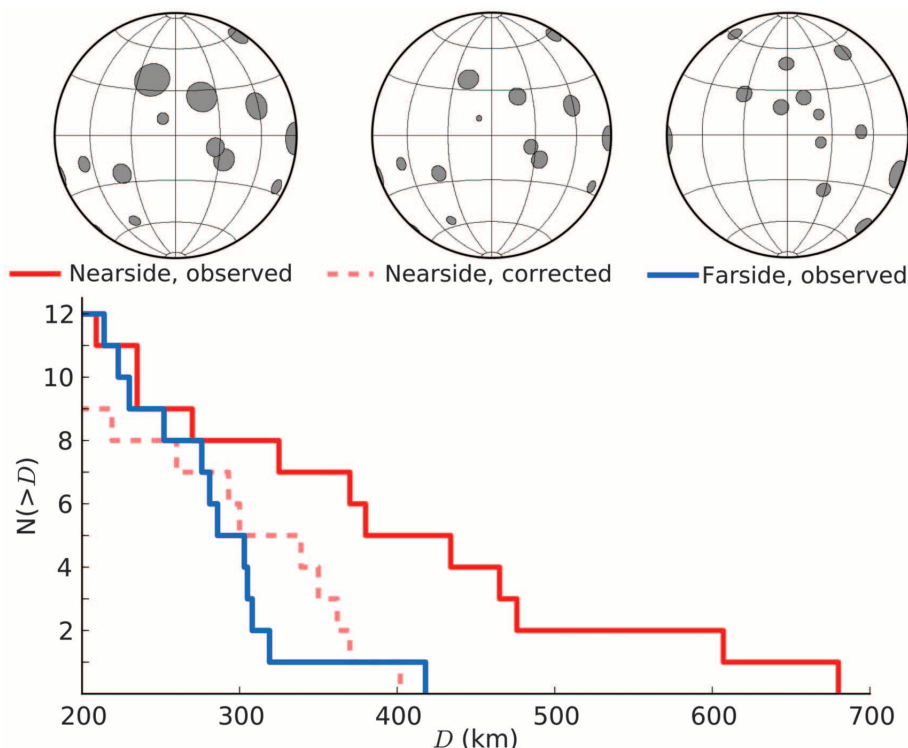


Fig. 2. Cumulative size distribution of observed lunar impact basins with diameters of crustal thinning D greater than 200 km for both hemispheres. The nearside is shown in solid red, the farside in solid blue. The size distribution of nearside basins after correction for lateral variations in target properties is shown in dashed red. Hemispherical maps depict the sizes and locations of basins used in the size-frequency distributions.

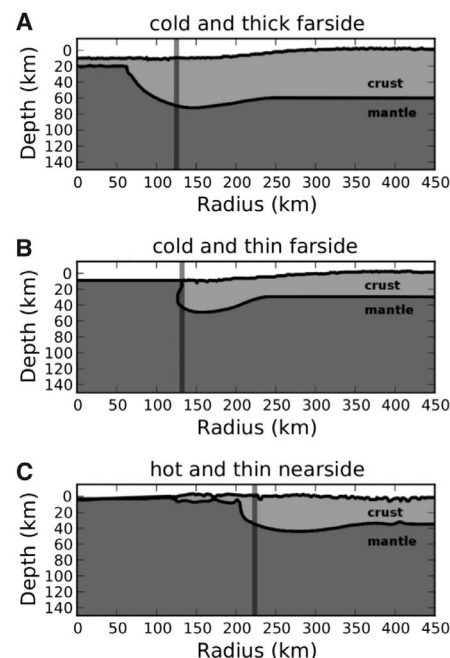


Fig. 3. Vertical cross sections of final surface topography and depth of the crust-mantle interface for three simulations of lunar impact basin formation. (A to C) Different temperature profiles for the farside [(A) and (B)] and nearside (C) correspond to the lunar thermal state at 4 Ga (M1/PKT1, fig. S2). Pre-impact crustal thicknesses were 60 km (A) and 30 km [(B) and (C)]. The basins were formed by the vertical impact of a 45-km-diameter projectile at an impact speed of 17 km s⁻¹ onto the Moon. The diameter of crustal thinning D shown by the vertical lines is the radial distance from basin center at which the crustal thickness reaches the pre-impact value.

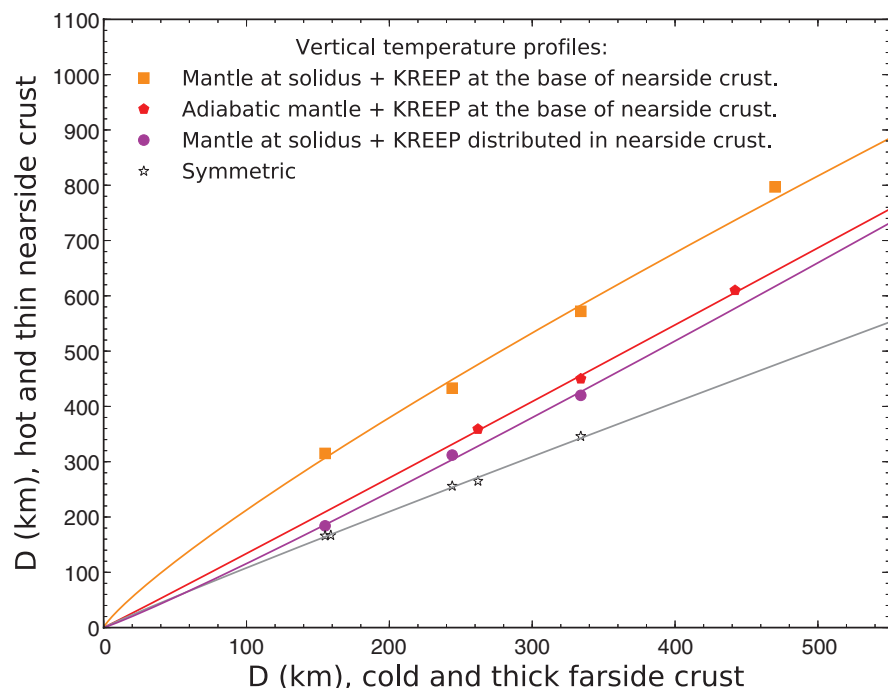


Fig. 4. Dependence of impact basin size on target properties. The ordinate is the diameter of crustal thinning D for a basin formed in a hot (KREEP-enriched) and 30-km-thick crust, and the abscissa is D for a basin formed in a cold and 60-km-thick crust, for the same size impactor. Points of the same color correspond to simulations with different projectile diameters. Variations for a given color reflect the different temperature profiles assumed for the nearside and farside; orange denotes the hottest temperature profile for the nearside (M1/PKT1, fig. S2) and violet denotes the coolest (M1/PKT2, fig. S2). The results in gray stars show D when the same temperature profile is used for both thin and thick crust.

the respective farside basins formed under similar conditions but in cold crust. These relationships between the diameter of crustal thinning on the nearside and farside hemispheres are largely independent of impact speed (from 10 to 17 km/s) and do not depend on differences in time of basin formation of up to a few hundred million years (fig. S3, table S4, and supplementary text).

The empirical relations from Fig. 4 can be used to compensate for the increase in basin size that results from lateral variations in target properties. Given that the absolute ages of most large lunar basins are poorly known, and that the subsurface temperature profile will vary both with time and distance from the PKT, such an exercise will be somewhat qualitative. We assumed that basins located within the PKT formed in the hottest and thinnest crust, and that basins surrounding the PKT formed in crust of intermediate temperature and thickness, and we then corrected the sizes of these basins to those that would be expected for impacts into the temperature regime of the colder farside highlands (supplementary text). Once lateral variations in target properties are included, Fig. 2 shows that the size distributions of impact basins on the nearside and farside hemispheres are comparable.

The concept of the late heavy bombardment (a spike in the impact cratering rate at ~4 Ga) (27–29) is based largely on the nearside impact basins that are either within or adjacent to the

PKT. The temperature profile beneath this region is not representative of the Moon as a whole, and the special nature of the lunar nearside implies that the magnitude of basin-forming impact bombardment has been overestimated, mainly with respect to the impactor mass flux. The size distribution of impact basins on the farside hemisphere of the Moon is a more accurate indicator of the impact history of the inner solar system than that on the nearside. Lateral variations in target properties could have affected the size distribution of impact basins on other planets, such as Mars, which possesses a marked dichotomy in crustal thickness between the northern lowlands and southern highlands. A different temperature profile, combined with lateral variations in crustal temperature (30), could be responsible for the lower density of large impact basins on Mercury (31) than on the Moon, and higher surface temperatures are likely to have played an important role in determining the final sizes of craters on Venus.

References and Notes

1. M. A. Wieczorek, R. J. Phillips, *Icarus* **139**, 246–259 (1999).
2. H. Hikida, M. A. Wieczorek, *Icarus* **192**, 150–166 (2007).
3. C. I. Fassett *et al.*, *J. Geophys. Res.* **117**, E00H06 (2012).
4. E. P. Turtle *et al.*, in *Large Meteorite Impacts III*, T. Kenkmann, F. Hörz, A. Deutsch, Eds. (Geological Society of America, Boulder, CO, 2005), pp. 1–24.
5. G. A. Neumann, M. T. Zuber, D. E. Smith, F. G. Lemoine, *J. Geophys. Res.* **101**, 16841–16863 (1996).

6. S. R. Bratt, S. C. Solomon, J. W. Head, C. H. Thurber, *J. Geophys. Res.* **90**, 3049–3064 (1985).
7. R. Potter, D. A. Kring, G. S. Collins, W. Kiefer, P. McGovern, *Geophys. Res. Lett.* **39**, L18203 (2012).
8. M. T. Zuber *et al.*, *Science* **339**, 668–671 (2013).
9. M. A. Wieczorek *et al.*, *Science* **339**, 671–675 (2013).
10. M. D. Norman, R. A. Duncan, J. J. Huard, *Geochim. Cosmochim. Acta* **74**, 763–783 (2010).
11. V. A. Fernandes, J. Fritz, B. P. Weiss, I. Garrick-Bethell, D. L. Shuster, *Meteorit. Planet. Sci.* **48**, 241–269 (2013).
12. M. Le Feuvre, M. A. Wieczorek, *Icarus* **214**, 1–20 (2011).
13. B. L. Jolliff, J. J. Gillis, L. A. Haskin, R. L. Korotev, M. A. Wieczorek, *J. Geophys. Res.* **105**, 4197–4216 (2000).
14. R. L. Korotev, *J. Geophys. Res.* **105**, 4317–4346 (2000).
15. M. A. Wieczorek, R. J. Phillips, *J. Geophys. Res.* **105**, 20417–20430 (2000).
16. S. C. Solomon, R. P. Comer, J. W. Head, *J. Geophys. Res.* **87**, 3975–3992 (1982).
17. S. Kamata *et al.*, *J. Geophys. Res.* **118**, 398–415 (2013).
18. S. Zhong, E. M. Parmentier, M. T. Zuber, *Earth Planet. Sci. Lett.* **177**, 131–140 (2000).
19. P. C. Hess, E. M. Parmentier, *J. Geophys. Res.* **106**, 28023–28032 (2001).
20. M. Laneuville, M. A. Wieczorek, D. Breuer, N. Tosi, *J. Geophys. Res.* **118**, 1435–1452 (2013).
21. A. A. Amsden, H. M. Ruppel, C. W. Hirt, *SALE: A Simplified ALE Computer Program for Fluid Flow at All Speeds* (Report LA-8095, Los Alamos National Laboratory, 1980).
22. G. S. Collins, H. J. Melosh, B. A. Ivanov, *Meteorit. Planet. Sci.* **39**, 217–231 (2004).
23. K. Wünnemann, G. S. Collins, H. J. Melosh, *Icarus* **180**, 514–527 (2006).
24. E. Pierazzo, N. A. Artemieva, B. A. Ivanov, in *Large Meteorite Impacts III*, T. Kenkmann, F. Hörz, A. Deutsch, Eds. (Geological Society of America, Boulder, CO, 2005), pp. 443–457.
25. B. A. Ivanov, H. J. Melosh, E. Pierazzo, in *Large Meteorite Impacts and Planetary Evolution IV*, R. L. Gibson, W. U. Reimold, Eds. (Geological Society of America, Boulder, CO, 2010), pp. 29–49.
26. C. Hüttig, K. Stemmer, *Phys. Earth Planet. Inter.* **171**, 137–146 (2008).
27. F. Tera, D. A. Papanastassiou, G. J. Wasserburg, *Earth Planet. Sci. Lett.* **22**, 1–21 (1974).
28. D. A. Kring, B. A. Cohen, *J. Geophys. Res.* **107**, 5009 (2002).
29. K. Tsiganis, R. Gomes, A. Morbidelli, H. F. Levison, *Nature* **435**, 459–461 (2005).
30. J.-P. Williams, J. Ruiz, M. A. Rosenburg, O. Aharonson, R. J. Phillips, *J. Geophys. Res.* **116**, E01008 (2011).
31. C. I. Fassett *et al.*, *J. Geophys. Res.* **117**, E00L08 (2012).
32. D. J. Lawrence *et al.*, *J. Geophys. Res.* **108**, 5102 (2003).

Acknowledgments: Supported by UnivEarthS LabEx project of the University of Sorbonne Paris Cité grants ANR-10-LABX-0023 and ANR-11-IDEX-0005-02 (K.M.), UK Science & Technology Facilities Council grant ST/J001260/1 (G.S.C.), and the French Space Agency (CNES). The GRAIL mission is supported by the Discovery Program of NASA and is performed under contract to the Massachusetts Institute of Technology and the Jet Propulsion Laboratory, California Institute of Technology. We gratefully acknowledge the developers of iSALE-2D/3D (www.isale-code.de).

Supplementary Materials

www.sciencemag.org/content/342/6159/724/suppl/DC1
Supplementary Text
Figs. S1 to S4
Tables S1 to S5
References (33–55)

15 July 2013; accepted 30 September 2013
10.1126/science.1243224

Th17 Cell Differentiation Is Regulated by the Circadian Clock

Xiaofei Yu,¹ Darcy Rollins,¹ Kelly A. Ruhn,¹ Jeremy J. Stubblefield,² Carla B. Green,² Masaki Kashiwada,^{3*} Paul B. Rothman,^{3†} Joseph S. Takahashi,^{2,4} Lora V. Hooper^{1,4‡}

Circadian clocks regulate numerous physiological processes that vary across the day-night (diurnal) cycle, but if and how the circadian clock regulates the adaptive immune system is mostly unclear. Interleukin-17-producing CD4⁺ T helper (Th17) cells are proinflammatory immune cells that protect against bacterial and fungal infections at mucosal surfaces. Their lineage specification is regulated by the orphan nuclear receptor ROR γ t. We show that the transcription factor NFIL3 suppresses Th17 cell development by directly binding and repressing the *Ror γ t* promoter. NFIL3 links Th17 cell development to the circadian clock network through the transcription factor REV-ERB α . Accordingly, Th17 lineage specification varies diurnally and is altered in *Rev-erb α* ^{-/-} mice. Light-cycle disruption elevated intestinal Th17 cell frequencies and increased susceptibility to inflammatory disease. Thus, lineage specification of a key immune cell is under direct circadian control.

The development and function of the immune system is profoundly affected by environmental factors such as microorganisms (1, 2), nutrients (3), and light cues (4). Interleukin-17 (IL-17A and F)-producing CD4⁺ T helper (Th17) cells are a key immune cell lineage that protects against bacterial and fungal infection (5) and is associated with inflammatory

disease (6). Th17 cell frequencies in the intestine are influenced by microbiota composition (2, 7), but few other environmental cues are known to regulate Th17 cell development.

NFIL3, also known as E4BP4, is a basic leucine zipper transcription factor that regulates a number of immune processes (8). *Nfil3* polymorphisms are associated with human inflammatory

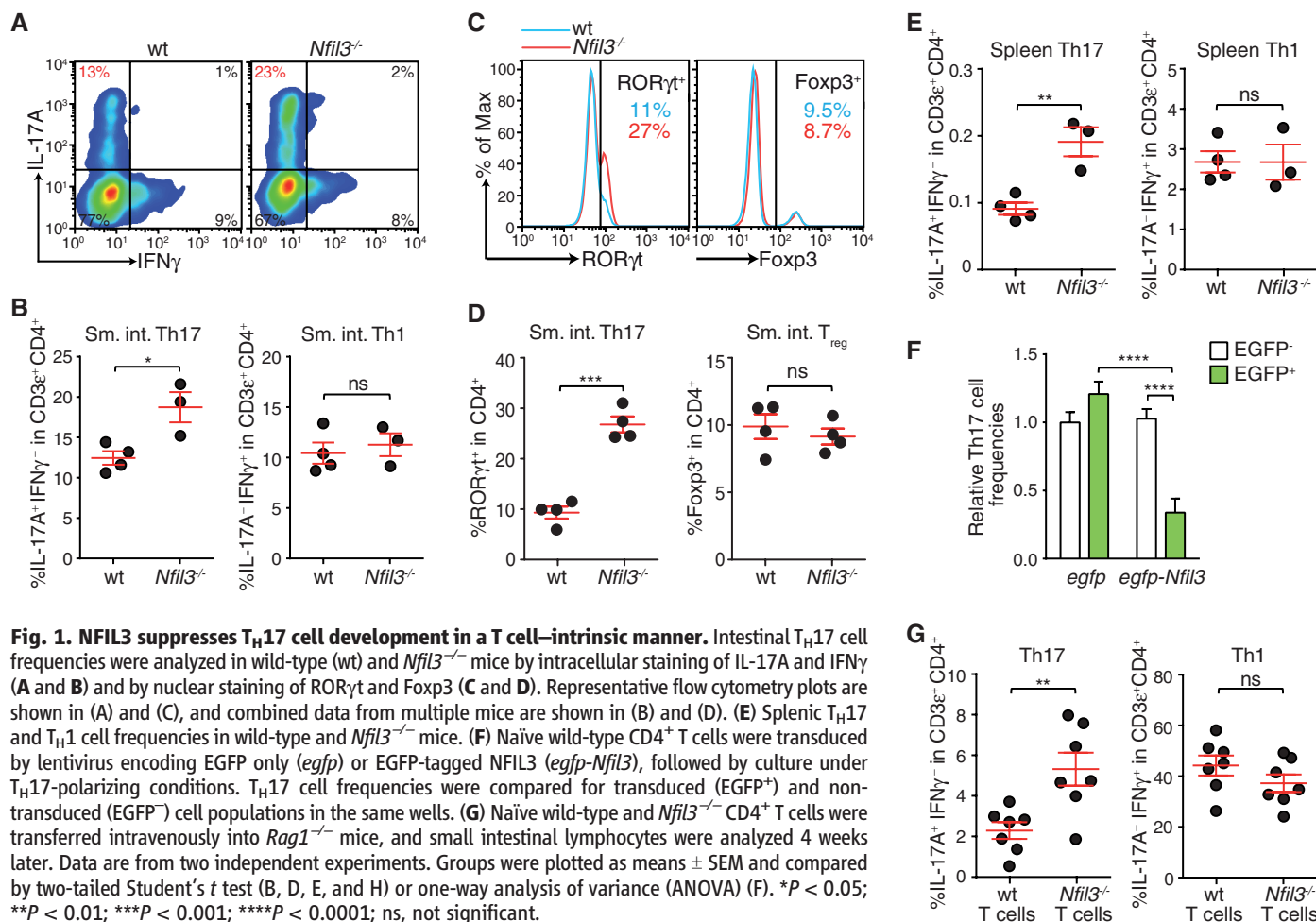
bowel disease (9). In agreement with this finding, ~10% of *Nfil3*^{-/-} mice, but none of the wild-type mice housed in our specific pathogen-free (SPF) barrier facility, exhibited rectal prolapse and immune cell infiltration into the intestine at 6 to 9 months of age (fig. S1, A and B). These abnormalities prompted us to examine CD4⁺ T cells, which are critical for intestinal immune homeostasis (10). *Nfil3*^{-/-} mice had higher IL-17A⁺ and ROR γ t⁺ Th17 cell frequencies than wild-type mice in both small intestine (Fig. 1, A to D) and colon (fig. S1, C and D). In contrast, there were no significant differences in interferon- γ -positive (IFN γ ⁺) Th1 (Fig. 1, A and B) or Foxp3⁺ regulatory T (T_{reg}) cell frequencies (Fig. 1, C and D), in agreement with prior findings (11). Thus, NFIL3

¹Department of Immunology, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ²Department of Neuroscience, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ³Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA. ⁴The Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

*Present address: Department of Biochemistry, Jichi Medical University, Shimotsuke, Tochigi 329-0498, Japan.

†Present address: The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

‡Corresponding author. E-mail: lora.hooper@utsouthwestern.edu



deficiency has an impact on intestinal T_H17 but not T_H1 or T_{reg} cell frequencies.

Because intestinal T_H17 cell development is sensitive to microbiota composition (2, 7), we considered whether the higher T_H17 frequencies in *Nfil3*^{-/-} mice were due to an altered microbiota. Microbiota transfer from conventionally raised wild-type and *Nfil3*^{-/-} mice into germ-free wild-type mice yielded similar intestinal T_H17 cell frequencies in the two groups of recipient mice (fig. S2, A and B), indicating that intestinal T_H17 cell expansion in *Nfil3*^{-/-} mice was not due to altered microbiota composition. This is consistent with the elevated T_H17 cell frequencies in the spleens of *Nfil3*^{-/-} mice (Fig. 1E), indicating that loss of NFIL3 leads to a systemic defect in suppression of T_H17 cell development. However, because microbiota composition and age are known to affect T_H17 cell frequencies (2, 7), we used age- and sex-matched littermates that were cocaged to minimize microbiota differences in all experiments.

To assess whether NFIL3 suppresses T_H17 development, we overexpressed enhanced green fluorescent protein (EGFP)-tagged NFIL3 in naïve CD4⁺ T cells by lentiviral transduction and grew cells under T_H17-polarizing conditions. Because only a fraction of the T cells became transduced, we were able to analyze both transduced (EGFP⁺) and nontransduced (EGFP⁻) cells in each sample. CD4⁺ T cells transduced with lentivirus encoding NFIL3 yielded lower T_H17 cell frequencies than nontransduced T cells (Fig. 1F and fig. S3). In contrast, transduced and nontransduced T cells yielded similar T_H17 cell frequencies when control lentivirus (*egfp* only) was used. Thus, NFIL3 suppresses T_H17 cell development in a T cell-intrinsic manner in vitro. Although a prior study found that retroviral transduction of *Nfil3* did not significantly affect T_H17 cell development (12), differences in the transduction protocol likely account for the different experimental outcomes.

To test whether NFIL3 has a T cell-intrinsic role in T_H17 development in vivo, we adoptively transferred naïve wild-type and *Nfil3*^{-/-} CD4⁺ T cells into lymphopenic *Rag1*^{-/-} mice (13). More *Nfil3*^{-/-} T cells differentiated into T_H17 cells than did wild-type T cells, which indicated that NFIL3 suppresses T_H17 cell development in a T cell-intrinsic manner (Fig. 1G and fig. S4A). In accordance with the pathogenic role of T_H17 cells in this model, *Rag1*^{-/-} mice receiving *Nfil3*^{-/-} T cells exhibited greater weight loss than mice receiving wild-type T cells (fig. S4B), as shown previously (11). IFN γ ⁺ T_H1 cell frequencies between the two groups of recipient mice were similar, which confirmed that NFIL3 preferentially affects T_H17 cell development (Fig. 1G).

T_H17 cell specification requires the orphan nuclear receptor ROR γ t (12, 14). Analysis of the *Roryt* promoter sequence revealed a putative NFIL3-binding site (GTTACTTAA) that was conserved between human and mouse (fig. S5). Accordingly, *Roryt* expression was higher in *Nfil3*^{-/-} T_H17 cells than in wild-type cells (Fig. 2A). A chro-

matin immunoprecipitation (ChIP) assay with an NFIL3-specific antibody (15) indicated that NFIL3 bound to the *Roryt* promoter in mouse CD4⁺ T cells (Fig. 2B). Binding of NFIL3 to the conserved GTTACTTAA motif was demonstrated by electrophoretic mobility shift assay (EMSA), and binding specificity of NFIL3 was further established by competition with unlabeled probes and supershift with the antibody against NFIL3 (Fig. 2C). Finally, overexpression of NFIL3 suppressed *Roryt* promoter activity in Jurkat T cells as measured by a luciferase reporter assay (Fig. 2D). Repression was dependent on the GTTACTTAA motif, as introduction of a point mutation (GTTACTTTA) abolished the repressive effect. Thus, NFIL3 binds to the GTTACTTAA motif in the *Roryt* promoter and represses promoter activity.

NFIL3 coordinates inputs from multiple regulatory pathways, including the circadian clock (16, 17). The circadian clock is an autoregulatory transcriptional network driven by the primary activators BMAL1 and CLOCK. It is negatively regulated by two feedback arms, one of which comprises the nuclear receptor REV-ERBa and its close homolog REV-ERB β (18). The circadian clock circuitry has been shown to function in CD4⁺ T cells (19). REV-ERBa directly represses *Nfil3* transcription by binding to a consensus sequence in the *Nfil3* gene locus (Fig. 3A

and fig. S6) (20). Accordingly, *Nfil3* expression was higher in activated *Rev-erba*^{-/-} CD4⁺ T cells than in wild-type cells (Fig. 3A) (21). Activated CD4⁺ T cells were used in this experiment because *Nfil3* mRNAs are more abundant in activated than in naïve CD4⁺ T cells (fig. S7A), consistent with the global increase in transcription in activated lymphocytes (22). Additionally, naïve *Rev-erba*^{-/-} CD4⁺ T cells showed a decreased capacity to differentiate into T_H17 cells when cultured under T_H17-polarizing conditions (Fig. 3B), and intestinal T_H17 cell frequencies were reduced in *Rev-erba*^{-/-} mice (Fig. 3, C and D). In contrast, no differences were observed in T_H1 cell frequencies (Fig. 3, C and D). Thus, T_H17 cell lineage specification is linked to the clock regulatory network through NFIL3 and REV-ERBa.

To confirm the role of the circadian clock in T_H17 development, we assessed *Clock* ^{Δ 19/ Δ 19} mice, which produce a dominant-negative CLOCK that inhibits the function of the BMAL1/CLOCK complex (23). BMAL1/CLOCK is required for REV-ERBa expression (Fig. 3E) (24), and accordingly, *Clock* ^{Δ 19/ Δ 19} mice exhibited higher *Nfil3* expression in activated CD4⁺ T cells (Fig. 3E), lowered capacity for T_H17 cell differentiation in naïve T cells (Fig. 3F), and reduced intestinal T_H17 cell frequencies when compared with wild-type mice (Fig. 3, G and H). Unlike *Rev-erba*^{-/-}

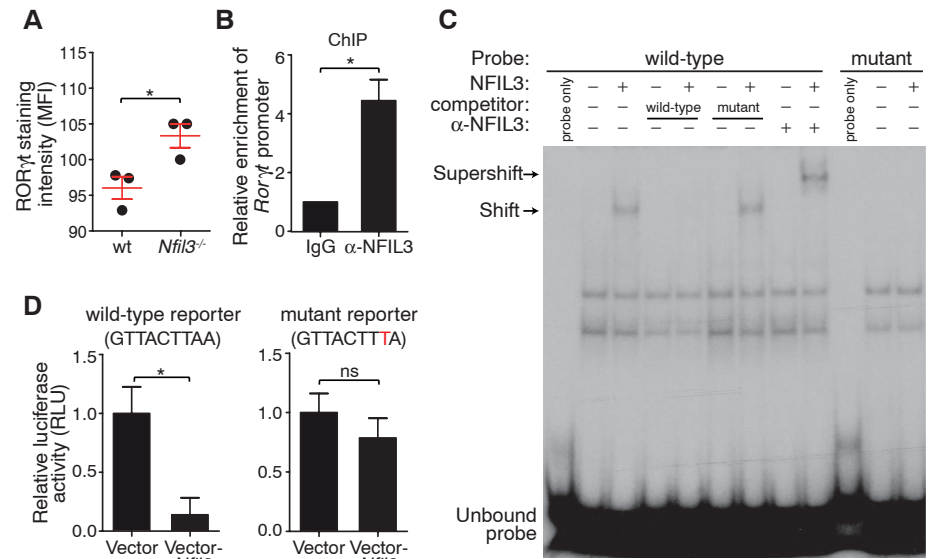


Fig. 2. NFIL3 represses *Roryt* transcription by binding directly to its promoter. (A) Lamina propria lymphocytes from wild-type and *Nfil3*^{-/-} mice were analyzed by nuclear staining of ROR γ t, and mean fluorescence intensities (MFI) were plotted. (B) ChIP analysis of CD4⁺ T cells using immunoglobulin G (IgG) or NFIL3-specific antibody. Enrichment of the *Roryt* promoter was calculated as the ratio of the NFIL3-specific antibody pull-down to the IgG control pull-down. (C) EMSA with nuclear extracts of human embryonic kidney 293T (HEK293T) cells transfected with an empty vector or an NFIL3-encoding vector. A 30-base pair (bp) DNA fragment encompassing the NFIL3-binding site from the *Roryt* promoter was synthesized as a wild-type probe. The mutant probe has the same sequence except that the NFIL3 binding site was mutated. NFIL3 binding specificity was demonstrated by competition with unlabeled probes and supershift with the NFIL3-specific antibody. (D) Luciferase reporter assay. A 1018-bp (from -1013 to +5) fragment of the *Roryt* promoter was fused with firefly luciferase, and position 8 of the NFIL3 binding site was mutated from A to T in the mutant reporter. Jurkat T cells were transfected with reporters and an empty vector or an NFIL3-encoding vector. Luciferase activity was normalized to cells transfected with vector-only controls. Groups were plotted as means $\pm$ SEM and compared by two-tailed Student's *t* test. **P* < 0.05; ns, not significant.

mice, *Clock*^{Δ19/Δ19} mice also exhibited lower intestinal T_H1 cell frequencies (Fig. 3, G and H), which suggested that the circadian clock also has an impact on other intestinal CD4⁺ T cell subsets such as T_H1 cells.

To examine T_H17 development during the circadian cycle, we housed age- and sex-matched mice under either normal light cycles (LD, 12-hour light: 12-hour dark) or reversed light cycles (DL, 12-hour dark: 12-hour light) (fig. S8A). We found that *Nfil3* expression was lower during the day and higher at night, whereas *Rorγt* expression was higher during the day and lower at night (Fig. 4, A and B). There were no significant differences in CD4⁺ T cell composition at these time points (fig. S8, B and C). The expression of *Nfil3* and *Rorγt* in opposite phases of the circadian cycle was consistent with diurnal variation in binding of NFIL3 to the *Rorγt* promoter (Fig. 4C). Accordingly, naïve CD4⁺ T cells isolated during the day were more likely to differentiate into T_H17 cells after in vitro polarization than those isolated at night (Fig. 4D). This difference was abolished in *Nfil3*^{−/−} cells (Fig. 4E), which showed that the diurnal variation in T_H17 lineage specification is NFIL3-dependent. Thus, T_H17 lineage specification is regulated in a diurnal manner and is synchronized across the T cell population by the circadian clock.

Consistent with the relatively long half-life (25) and week-long differentiation process of T_H17 cells, we found that intestinal T_H17 cell frequencies were unaltered during a single 24-hour cycle (fig. S9). We therefore tested whether circadian disruption by chronic light-cycle perturbations altered T_H17 cell frequencies (Fig. 4F). T_H17 cell frequencies were higher in the intestines and spleens of mice subjected to perturbed light cycles as compared with mice maintained under a normal light cycle (Fig. 4G and fig. S10A), with no significant impact on cell proliferation or cell survival (fig. S11). This was coincident with a decrease in intestinal T_H1 cell frequencies (fig. S10B), but no decrease in spleen T_H1 cell frequencies (fig. S10A). Microbiota from mice under normal and perturbed light cycles yielded similar intestinal T_H17 cell frequencies when transplanted into germ-free recipients (fig. S12, A and B), which suggested that T_H17 cell expansion was not due to altered microbiota composition. The increase in T_H17 cell frequencies was suppressed in *Rev-erba*^{−/−} and, to a larger extent, in *Rev-erba*^{−/−}β^{−/−} (double knockout) mice (Fig. 4G), which indicated that the increased T_H17 cell frequencies required REV-ERBα/β and were unlikely to arise from nonspecific effects of light-cycle perturbation. Mice subjected to perturbed light cycles were more susceptible to dextran sulfate

sodium (DSS)-induced colitis, as measured by weight loss and colon shortening (Fig. 4, H to K). The enhanced pathology could be ameliorated by neutralizing IL-17A (Fig. 4, H to K), which suggested that the increased susceptibility to DSS treatment was due in part to the elevated T_H17 cell frequencies in the mice subjected to perturbed light cycles. Thus, chronic light-cycle perturbation leads to elevated T_H17 cell frequencies in the intestine and enhanced susceptibility to inflammatory disease. This suggests that diurnal regulation of T_H17 cell differentiation is important for maintaining homeostatic T_H17 cell frequencies and restraining inflammation.

Together, our results demonstrate that NFIL3 suppresses T_H17 cell development by directly repressing *Rorγt* transcription and links T_H17 cell development to the circadian clock (Fig. 4K). This ensures that T_H17 lineage specification preferentially occurs at a specific stage of the circadian cycle and is thus synchronized across the entire T cell population. We suggest that over-accumulation of T_H17 cells may be limited by ensuring that all T cells within a population traverse this critical developmental checkpoint in synchrony rather than at random times during the day-night cycle.

Modern life often involves chronic circadian disruptions, such as night shift work or jet lag,

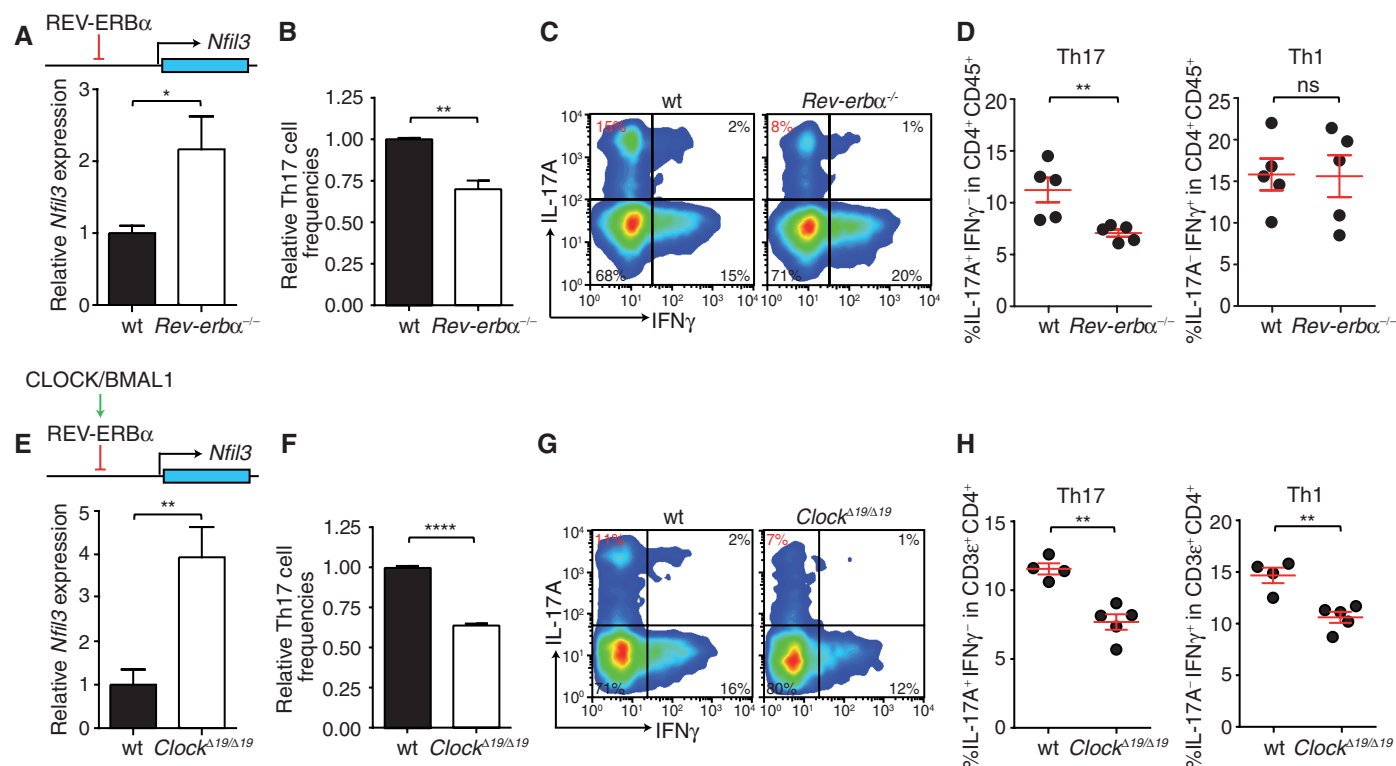
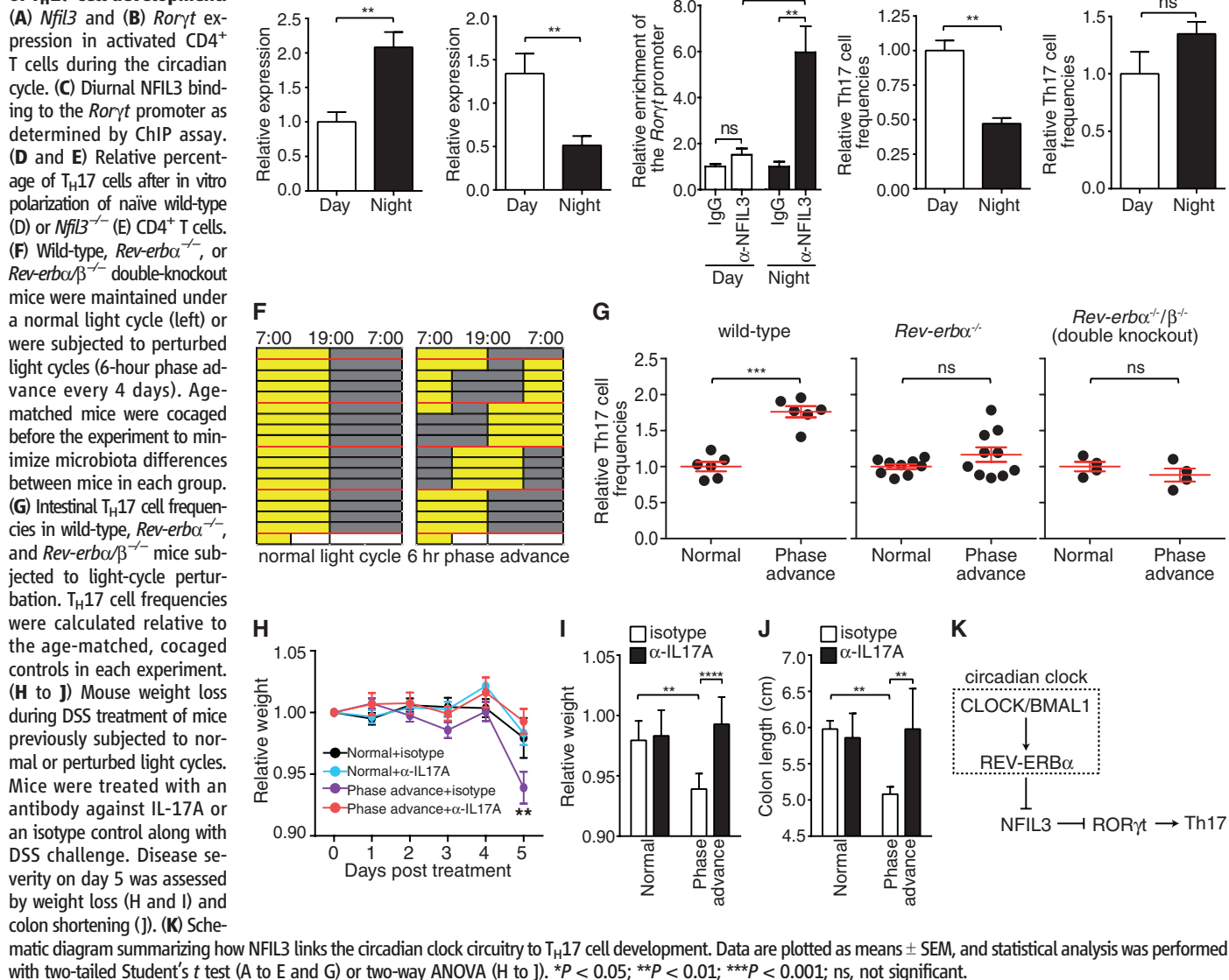


Fig. 3. T_H17 cell development is regulated by the circadian clock transcriptional network. (A) *Nfil3* expression levels were quantified in activated CD4⁺ T cells by real-time polymerase chain reaction. (B) Naïve wild-type and *Rev-erba*^{−/−} CD4⁺ T cells were polarized to T_H17 cells in vitro. (C and D) Small intestinal T_H17 cell frequencies in wild-type and *Rev-erba*^{−/−} mice. Representative flow cytometry plots are shown in (C) and combined data are shown in (D). (E) *Nfil3* expression in activated wild-type and

Clock^{Δ19/Δ19} CD4⁺ T cells. (F) Naïve wild-type and *Clock*^{Δ19/Δ19} CD4⁺ T cells were cultured under T_H17-polarizing conditions. (G and H) Small intestinal T_H17 cell frequencies of wild-type and *Clock*^{Δ19/Δ19} mice. Representative flow cytometry plots are shown in (G), and combined data are shown in (H). Data are plotted as means ± SEM, and statistics were performed with the two-tailed Student's *t* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant.

Fig. 4. Diurnal regulation of T_H17 cell development.



that are linked to human inflammatory diseases (26, 27). Our findings suggest that the pathologic consequences of circadian disruption may be due in part to direct interactions between the circadian clock and the pathways that regulate proinflammatory immune cell development.

References and Notes

- H. L. Cash, C. V. Whitham, C. L. Behrendt, L. V. Hooper, *Science* **313**, 1126–1130 (2006).
- I. I. Ivanov et al., *Cell* **139**, 485–498 (2009).
- E. A. Kiss et al., *Science* **334**, 1561–1565 (2011).
- A. Arjona, A. C. Silver, W. E. Walker, E. Fikrig, *Trends Immunol.* **33**, 607–612 (2012).
- T. Korn, E. Bettelli, M. Oukka, V. K. Kuchroo, *Annu. Rev. Immunol.* **27**, 485–517 (2009).
- K. J. Maloy, M. C. Kullberg, *Mucosal Immunol.* **1**, 339–349 (2008).
- I. I. Ivanov et al., *Cell Host Microbe* **4**, 337–349 (2008).
- V. Male, I. Nisoli, D. M. Gascoyne, H. J. Brady, *Trends Immunol.* **33**, 98–102 (2012).
- L. Jostins et al., *Nature* **491**, 119–124 (2012).
- L. V. Hooper, A. J. Macpherson, *Nat. Rev. Immunol.* **10**, 159–169 (2010).
- Y. Motomura et al., *Nat. Immunol.* **12**, 450–459 (2011).
- M. Ciofani et al., *Cell* **151**, 289–303 (2012).
- D. V. Ostainin et al., *Am. J. Physiol. Gastrointest. Liver Physiol.* **296**, G135–G146 (2009).
- I. I. Ivanov et al., *Cell* **126**, 1121–1133 (2006).
- M. Kashiwada et al., *Proc. Natl. Acad. Sci. U.S.A.* **107**, 821–826 (2010).
- N. Koike et al., *Science* **338**, 349–354 (2012).
- H. R. Ueda et al., *Nat. Genet.* **37**, 187–192 (2005).
- J. A. Mohawk, C. B. Green, J. S. Takahashi, *Annu. Rev. Neurosci.* **35**, 445–462 (2012).
- T. Bollinger et al., *PLOS ONE* **6**, e29801 (2011).
- H. Duez et al., *Gastroenterology* **135**, 689–698, 698.e5 (2008).
- N. Preitner et al., *Cell* **110**, 251–260 (2002).
- F. Kouzine et al., *Cell* **153**, 988–999 (2013).
- D. P. King et al., *Cell* **89**, 641–653 (1997).
- G. Triqueneaux et al., *J. Mol. Endocrinol.* **33**, 585–608 (2004).
- M. Pepper et al., *Nat. Immunol.* **11**, 83–89 (2010).
- D. A. Bechtold, J. E. Gibbs, A. S. Loudon, *Trends Pharmacol. Sci.* **31**, 191–198 (2010).
- F. A. Scheer, M. F. Hilton, C. S. Mantzoros, S. A. Shea, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4453–4458 (2009).

Acknowledgments: We thank C. Behrendt, C. Clements, and S. Murray for assistance with mouse experiments. M. Izumo provided advice and assistance with the circadian cycle experiments, and B. Li for help with the EMSA. We also thank F. Yarovinsky for critical reading of the manuscript. The data presented in this manuscript are tabulated in the main paper and the supplementary materials. The MODE-K cells were supplied to the authors under a materials transfer agreement from Inserm. J.S.T. is cofounder, scientific advisory board member and paid consultant of Reset Therapeutics, Inc., a biotech company working on circadian rhythms and metabolism. This work was supported by NIH R01 DK070855 (L.V.H.), a Burroughs Wellcome Foundation New Investigators in the Pathogenesis of Infectious Diseases Award (L.V.H.), and the Howard Hughes Medical Institute (J.S.T. and L.V.H.).

Supplementary Materials

www.sciencemag.org/content/342/6159/727/suppl/DC1
 Materials and Methods
 Figs. S1 to S12
 References (28–30)

29 July 2013; accepted 9 October 2013
 10.1126/science.1243884

High-Resolution Mapping of the Spatial Organization of a Bacterial Chromosome

Tung B. K. Le,¹ Maxim V. Imakaev,² Leonid A. Mirny,^{2,3*} Michael T. Laub^{1,4*}

Chromosomes must be highly compacted and organized within cells, but how this is achieved in vivo remains poorly understood. We report the use of chromosome conformation capture coupled with deep sequencing (Hi-C) to map the structure of bacterial chromosomes. Analysis of Hi-C data and polymer modeling indicates that the *Caulobacter crescentus* chromosome consists of multiple, largely independent spatial domains that are probably composed of supercoiled plectonemes arrayed into a bottle brush–like fiber. These domains are stable throughout the cell cycle and are reestablished concomitantly with DNA replication. We provide evidence that domain boundaries are established by highly expressed genes and the formation of plectoneme-free regions, whereas the histone-like protein HU and SMC (structural maintenance of chromosomes) promote short-range compaction and the colinearity of chromosomal arms, respectively. Collectively, our results reveal general principles for the organization and structure of chromosomes in vivo.

In all organisms, chromosomal DNA must be compacted by nearly three orders of magnitude to fit within the limited volume of a cell. Chromosomes must adopt structures that are compatible with critical cellular processes such as

transcription, DNA replication, and chromosome segregation. Although bacterial chromosomes are probably highly organized within cells (1–6), the resolution of previous studies has been limited. For eukaryotes, chromosome conformation cap-

ture coupled with deep sequencing, or Hi-C, has enabled higher-resolution studies of chromosome structure in vivo (7, 8). These studies have suggested that interphase chromosomes are organized into a series of topological or structural domains <1 Mb in size (8–11), but the factors that create, maintain, and influence these domains are presently unknown.

To study the organization of bacterial chromosomes with high resolution, we used Hi-C on *Caulobacter* cells (figs. S1 and S2). We performed Hi-C on swarmer cells that each contain a single circular and unreplicated chromosome. To analyze our Hi-C data, we divided the genome into 10-kilobase (kb) bins, with interaction frequencies for each restriction fragment assigned to corresponding bins. We visualized interactions as a heat map where each matrix position, m_{ij} , reflects the relative frequency of interactions be-

¹Department of Biology, Massachusetts Institute of Technology (MIT), Cambridge, MA 02139, USA. ²Department of Physics, MIT, Cambridge, MA 02139, USA. ³Institute for Medical Engineering and Sciences, MIT, Cambridge, MA 02139, USA. ⁴Howard Hughes Medical Institute, MIT, Cambridge, MA 02139, USA.

*Corresponding author. E-mail: laub@mit.edu (M.T.L.); leonid@mit.edu (L.A.M.)

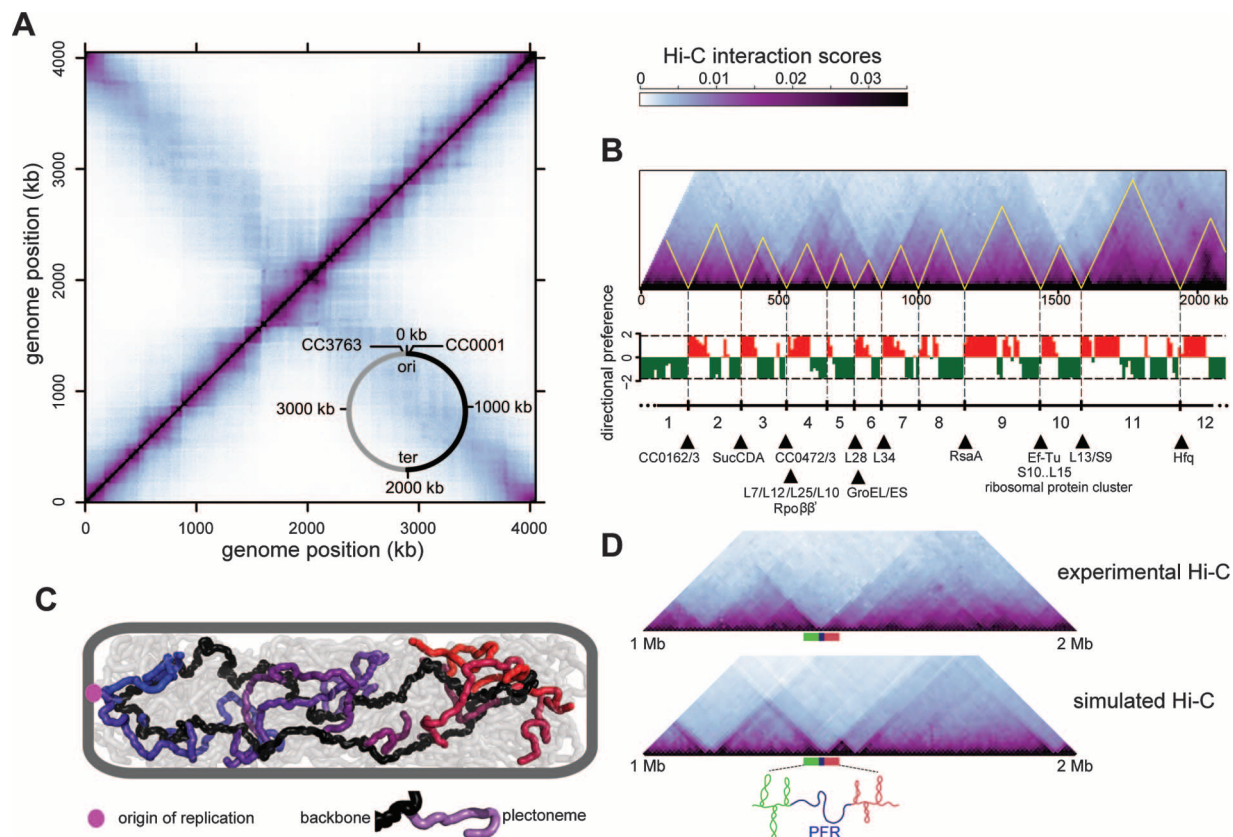


Fig. 1. Partitioning of the *Caulobacter* chromosome into CIDs. (A) Normalized *Nco*I Hi-C contact map for *Caulobacter* swarmer cells displaying contact frequencies for pairs of 10-kb bins across the genome. Axes indicate the genome position of each bin. (Inset) Simplified genomic map showing the origin of replication (*ori*) and terminus (*ter*), along with the right (black) and left (gray) chromosomal arms. (B) Hi-C contact map for one arm of the chromosome rotated 45° clockwise with directional preference plots below. Left-

and rightward preferences are shown as green and red bars, respectively. CIDs are outlined in yellow and numbered. Highly expressed genes at CID boundaries are listed (hypothetical genes are designated by GenBank ID no.). (C) Polymer chromosome model showing the polarly anchored origin (magenta), chromosome backbone (black), and plectonemes (gray, with every 10th plectoneme on one arm in a color). (D) Comparison of experimental and simulated Hi-C contact maps, indicating that PFRs can account for CIDs.

tween loci in bins i and j . For a description of data processing, normalization, reproducibility, and comparability to a previous 5C study (4), see the supplementary materials (figs. S3 to S6).

The swarmer cell interaction matrix contains two prominent diagonals (Fig. 1A). The main diagonal reflects high-frequency interactions between loci on the same chromosomal arm. The other, less prominent diagonal captures lower-frequency inter-arm contacts; i.e., those between loci on one chromosomal arm and those on the opposite arm of the circular genome. These locus pairs are separated by substantial distances in the primary genome sequence, but the Hi-C data indicate that they are often physically adjacent and capable of interacting. This overall pattern, also seen with 5C data (4), is consistent with the *Caulobacter* chromosome adopting an elongated structure, with the single origin anchored at one pole and the two chromosome arms running the length of the cell in close proximity.

Further inspection of the Hi-C interaction matrix revealed highly self-interacting regions, or chromosomal interaction domains (CIDs), of the genome that appear as squares along the main diagonal (Fig. 1A) or as triangles if the contact map is rotated 45° clockwise (Fig. 1B and figs. S7 and S8). Loci within a CID interact preferentially with other loci within the same CID as compared to other CIDs. Loci at the border of each CID strongly favor interactions with loci on their left- or righthand side, but not both, whereas loci in the middle of a CID show high levels of interaction with loci on both sides. The Hi-C matrix exhibited variability in boundary sharpness and some nested domains (Fig. 1B and figs. S8 and S9). This hierarchical organization resembles the so-called topologically associated domains (TADs) previously observed in eukaryotic Hi-C data (8–11).

To systematically map the boundaries of CIDs, we generated plots of directional preference as a function of genome position (Fig. 1B and figs. S7 to S9). There were 23 CIDs, ranging in length from 30 to 420 kb (table S1). CIDs were not artefacts of restriction site or sequencing-read densities (fig. S10) and were independently verified with a recombination-based assay for interaction frequencies (fig. S11). The CIDs identified must be present in most cells, because Hi-C reflects interactions in a population of cells. Individual cells could have other, perhaps transient, domains.

CID boundaries were enriched in highly expressed genes ($P = 7.7 \times 10^{-5}$, Fisher's exact test, fig. S10). Of the 23 CID boundaries, 17 contained one or more highly transcribed genes (Fig. 1B and figs. S8 and S10). We hypothesized that high gene expression unwinds the DNA duplex and creates plectoneme-free regions (PFRs), which form barriers between CIDs. These PFRs probably prevent the diffusion of supercoils and physically separate CIDs, thereby decreasing the contact probabilities of loci in different domains, as also suggested in *Salmonella* (1).

To better understand the three-dimensional organization of the *Caulobacter* chromosome, we developed a detailed polymer model (figs. S12 to S15). The chromosome was modeled as a circular polymer comprising a dense array of plectonemes that have no sequence specificity and are stochastic in length and location (Fig. 1C and fig. S12). We generated an equilibrium ensemble of chromosome conformations, simulated the Hi-C procedure on 25,000 modeled chromosomes, and compared the resulting data to experimental Hi-C data. By systematically varying model parameters, we identified values that provided the best fit to the observed Hi-C contact frequencies (figs. S13 and S14).

Our model reflects two broad levels of chromosomal organization. On one level, the DNA is arranged into a fiber of ~300 plectonemes separated by small spacers, resembling a bottle brush. Plectonemes ~15 kb in length separated by less than 300 bp provided the best agreement to Hi-C data. At a higher level, the bottle brush fiber forms a circular chromosome tethered at the pole by an origin-proximal region with chromosomal arms in close proximity down the long axis of the cell. We also used the model to examine the effects of PFRs on interactions between loci. A single PFR of ~2 kb created a space of ~100 to 200 nm between flanking loci. This spacer reduced contacts between neighboring plectonemes and prevented the diffusion of supercoils through the PFR in the simulations, recapitulating a CID boundary (Fig.

1D and fig. S16). We then introduced PFRs into the chromosome model at the locations of the 20 most highly expressed genes. Simulated Hi-C data generated a pattern of CIDs that resembled those observed experimentally, supporting the hypothesis that PFRs can induce CIDs (Fig. 1D and fig. S17).

To probe the role of gene expression in chromosome structure, we performed Hi-C on swarmer cells treated for 30 min with rifampicin (rif), an inhibitor of transcription elongation (12). The interaction matrix of rif-treated cells was globally similar to that of untreated cells, indicating that the overall shape of the chromosome was unperturbed (Fig. 2, A and B). However, CID boundaries were severely disrupted in rif-treated cells, leading to a nearly domain-free organization (Fig. 2B and figs. S18 and S19). Simulations of rif-treated chromosomes, performed by removing PFRs, also produced domain-free contact maps (figs. S19 to S21).

We also moved the highly expressed gene *rsaA* to the *vanA* locus, a poorly expressed region of the genome (fig. S22). The native *vanA* locus normally resides within a CID, but the insertion of *rsaA* generated a sharp new CID boundary at this position in the genome (Fig. 2C). Relocating *rsaA* to the *xytX* locus, ~1.7 Mb from the *vanA* locus, also created a new CID boundary at this location (fig. S23). We conclude that highly expressed genes play a direct role in defining chromosomal domain boundaries.

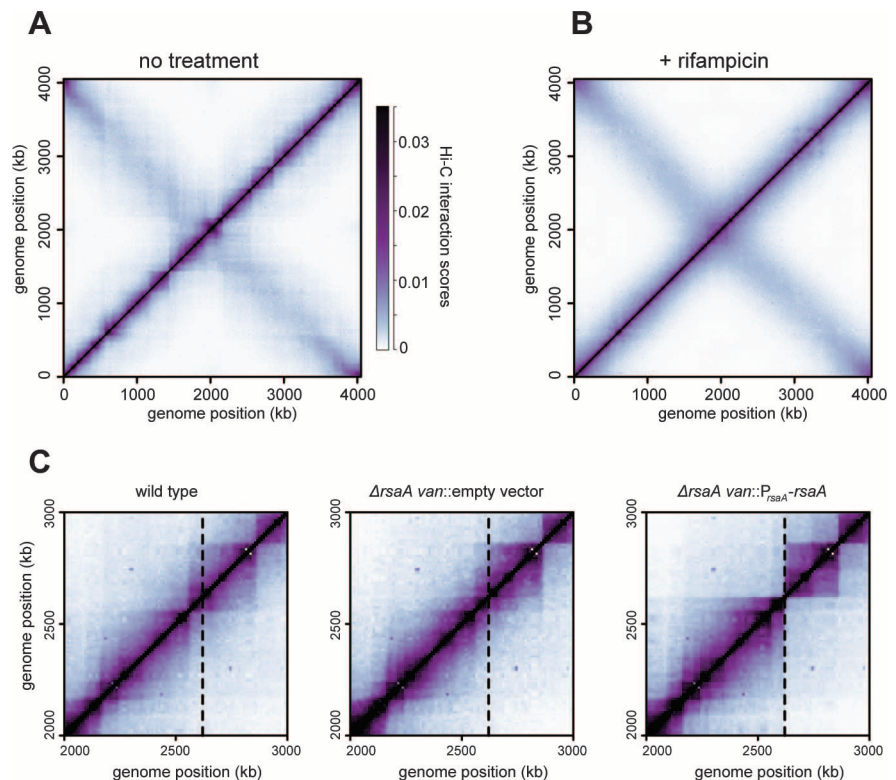


Fig. 2. Effect of inhibiting transcription on CID boundaries. Normalized BglII Hi-C contact maps for (A) untreated and (B) rif-treated swarmer cells. (C) Hi-C contact maps for wild-type, Δ *rsaA*, and Δ *rsaA* + *van::P_{rsaA}-rsaA* cells. Only the region of the genome containing the *van* locus (dashed line) is shown.

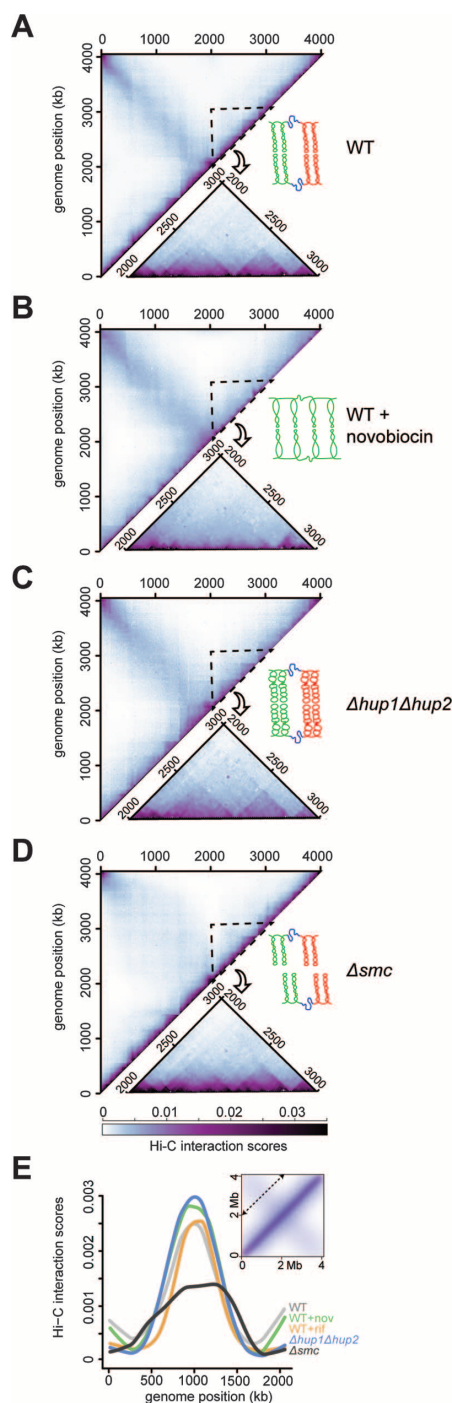


Fig. 3. Contribution of supercoiling, HU, and SMC to chromosome organization. Normalized BglII Hi-C contact maps for (A) wild-type, (B) novobiocin-treated wild-type, (C) $\Delta hup1\Delta hup2$, and (D) Δsmc swarmer cells. Only the upper left regions of the symmetric Hi-C maps are shown. A region from 2 to 3 Mb is enlarged and shown below each map. A cartoon summarizing the effects of each perturbation is shown, with neighboring domains of plectonemic DNA in red and green and plectoneme-free regions in blue. (E) Hi-C scores for the diagonal (indicated in the inset) of each contact map.

We also used Hi-C to probe the effect of inhibiting supercoiling on chromosomal organization. Swarmer cells were incubated for 30 min with a sublethal dose of novobiocin (fig. S24) and then subjected to Hi-C analysis (Fig. 3, A and B, and fig. S18). Novobiocin, which inhibits DNA gyrase and negative supercoiling (13), significantly reduced the frequency of interactions in the 20- to 200-kb range while modestly increasing interactions in the 200- to 800-kb range relative to the untreated wild type (fig. S25). Additionally, novobiocin reduced the sharpness and positions of CID boundaries (Fig. 3B and fig. S18). The decrease in interaction frequencies in the 20- to 200-kb range does not result from the emergence of a subpopulation of cells with gross defects in chromosome organization (fig. S26).

To model the effect of novobiocin, we increased the spacing between duplexes in a plectoneme monomer and increased the average spacing between plectonemes fivefold. Subsequent simulations reproduced the partial loss of CID boundaries and changes in contact frequencies observed (figs. S19, S20, and S27). Taken together, our results demonstrate that supercoiling is (i) critical to genome compaction in the 20- to 200-kb range and (ii) helps establish CIDs in vivo.

To investigate the role of nucleoid-associated proteins in chromosomal organization, we focused on the histone-like proteins HU1 and HU2 (14, 15). We isolated swarmer cells from a $\Delta hup1\Delta hup2$ strain and performed Hi-C. The interaction matrix for $\Delta hup1\Delta hup2$ was grossly similar to that of wild-type cells (Fig. 3C). The correlation between directional preferences for each bin along the chromosome of wild-type and $\Delta hup1\Delta hup2$ cells was high ($r = 0.73$, $P < 10^{-15}$), indicating that CID boundaries were retained, although they became less pronounced in the absence of HU1

and HU2 (fig. S28). The contact probability plot for $\Delta hup1\Delta hup2$ cells revealed a significant decrease in short-range contacts, up to ~100 kb, compared to the wild type (fig. S25). These changes suggest that deleting HU may disrupt interactions within and between neighboring plectonemes without affecting interplectoneme spacing, which is critical for producing CID boundaries (Fig. 3C and fig. S20). We modeled the effect of deleting HU by increasing the spacing between duplexes within a plectoneme; simulations based on this model recapitulated the Hi-C data (fig. S29). These analyses indicate that HU facilitates local short-range compaction of the genome, possibly through the packing and stabilization of plectonemic DNA.

SMC (structural maintenance of chromosomes) homologs are found in all domains of life and can form ringlike structures that facilitate chromosome cohesion or compaction (16–18). Hi-C analysis of *Caulobacter* Δsmc swarmer cells showed a clear drop in the frequency of inter-chromosomal arm interactions (Fig. 3, D and E). Concomitantly, loci typically interacted with a wider range of loci on the opposite chromosomal arm than did wild-type cells. In contrast, the frequencies of intra-arm interactions (fig. S25) and CID boundaries (fig. S28) were largely unaffected in the absence of SMC. Although many bacterial SMC proteins may compact DNA (19, 20), our data suggest that *Caulobacter* SMC contributes primarily to the colinearity of chromosome arms in swarmer cells.

To study chromosome organization changes during cell cycle progression, we performed Hi-C on synchronized cells collected at regular intervals during the cell cycle (Fig. 4). After replication initiation in *Caulobacter*, one origin remains near the stalked pole while the other

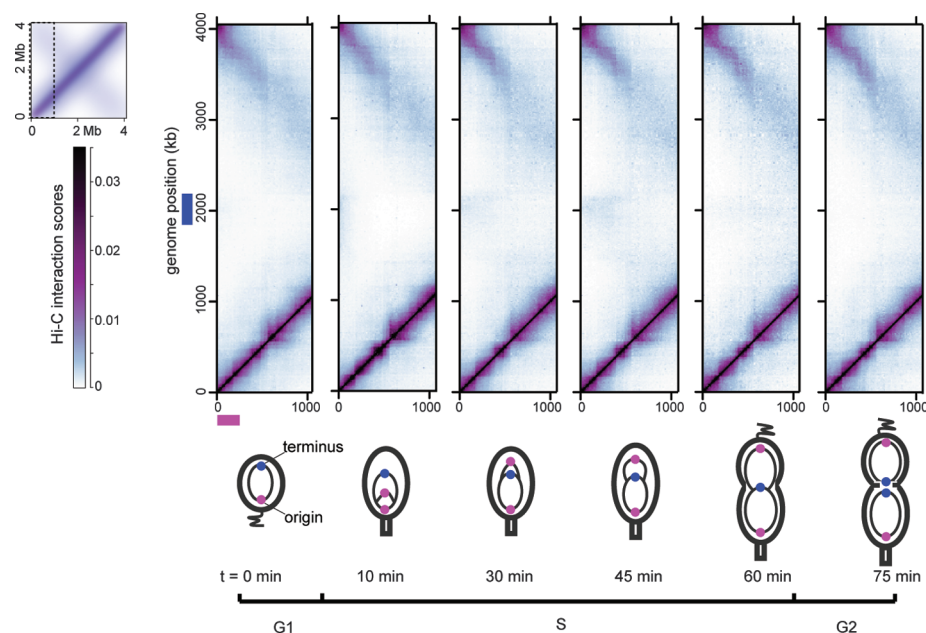


Fig. 4. Dynamics of chromosomal organization during cell cycle progression. Plots show a section of the Hi-C interaction map for the cell cycle time points indicated.

moves to the opposite pole (6). As the cell cycle progressed, the Hi-C contact maps indicated progressively more interactions between origin- and terminus-proximal loci (Fig. 4 and fig. S30), particularly at the 30- and 45-min time points, when the newly translocated origin is close to the polarly localized terminus (Fig. 4). However, the frequency of these interactions was still nearly 16-fold less than an average interaction between loci within 100 kb. This difference implies that the translocating chromosome is largely insulated from the anchored chromosome despite their physical proximity, which is likely to be advantageous to the segregation process by preventing the entanglement of chromosomes destined for different daughter cells. Also, the CIDs identified in swarmer cells (Fig. 1, A and B) remained intact throughout the cell cycle (Fig. 4), indicating that CIDs must get reestablished concurrently with, or shortly after, DNA replication (figs. S31 and S32), which may also help keep newly replicated chromosomes from becoming entangled.

CIDs are reminiscent of the TADs documented in eukaryotes (9–11, 21), suggesting that domains on a 100-kb length scale are a fundamental unit of chromosome structure in all organisms. Like TADs, *Caulobacter* CIDs often appear as nested domains, which may be related to the megabase-scale macrodomains identified in *Escherichia coli* (3, 5). The identification of CIDs indicates that many domain barriers in *Caulobacter* are relatively fixed; however, within each CID there could be additional barriers that arise and dissipate,

perhaps stochastically, as suggested in *Salmonella* and *E. coli* (1, 22), and CID boundaries may change in different growth conditions. Although chromosomal domains have now been documented in several organisms, the factors determining domain boundaries had been unclear. Although the DNA binding proteins HU and SMC contribute to chromosome organization, they do not significantly affect CIDs. Instead, our work points to supercoiling and highly expressed genes as critical determinants of domain formation in bacteria, and we suspect that similar mechanisms contribute to creating TADs in higher organisms, where boundaries may also be enriched in highly expressed genes (9).

References and Notes

1. B. M. Booker, S. Deng, N. P. Higgins, *Mol. Microbiol.* **78**, 1348–1364 (2010).
2. J. K. Fisher et al., *Cell* **153**, 882–895 (2013).
3. H. Niki, Y. Yamaichi, S. Hiraga, *Genes Dev.* **14**, 212–223 (2000).
4. M. A. Umbarger et al., *Mol. Cell* **44**, 252–264 (2011).
5. M. Valens, S. Penaud, M. Rossignol, F. Cornet, F. Boccard, *EMBO J.* **23**, 4330–4341 (2004).
6. P. H. Viollier et al., *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9257–9262 (2004).
7. J. Dekker, K. Rippe, M. Dekker, N. Kleckner, *Science* **295**, 1306–1311 (2002).
8. E. Lieberman-Aiden et al., *Science* **326**, 289–293 (2009).
9. J. R. Dixon et al., *Nature* **485**, 376–380 (2012).
10. E. P. Nora et al., *Nature* **485**, 381–385 (2012).
11. T. Sexton et al., *Cell* **148**, 458–472 (2012).
12. E. A. Campbell et al., *Cell* **104**, 901–912 (2001).
13. M. Gellert, M. H. O'Dea, T. Itoh, J. Tomizawa, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4474–4478 (1976).

14. S. C. Dillon, C. J. Dorman, *Nat. Rev. Microbiol.* **8**, 185–195 (2010).
15. F. Guo, S. Adhya, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 4309–4314 (2007).
16. A. Badrinarayanan, R. Reyes-Lamothe, S. Uphoff, M. C. Leake, D. J. Sherratt, *Science* **338**, 528–531 (2012).
17. T. Hirano, *Nat. Rev. Mol. Cell Biol.* **7**, 311–322 (2006).
18. M. A. Schwartz, L. Shapiro, *Mol. Microbiol.* **82**, 1359–1374 (2011).
19. P. L. Graumann, T. Knust, *Chromosome Res.* **17**, 265–275 (2009).
20. A. Volkov, J. Mascarenhas, C. Andrei-Selmer, H. D. Ulrich, P. L. Graumann, *Mol. Cell Biol.* **23**, 5638–5650 (2003).
21. C. Hou, L. Li, Z. S. Qin, V. G. Corces, *Mol. Cell* **48**, 471–484 (2012).
22. L. Postow, C. D. Hardy, J. Arsuaga, N. R. Cozzarelli, *Genes Dev.* **18**, 1766–1779 (2004).

Acknowledgments: Hi-C data were deposited in the Gene Expression Omnibus (accession no. GSE45966). We thank M. Umbarger for help in optimizing Hi-C and G. Fudenberg, A. Goloborodko, M. Hu, and T. Maxwell for discussions. M.T.L. is an Early Career Scientist of the Howard Hughes Medical Institute. T.B.K.L. is a Gordon and Betty Moore Foundation postdoctoral fellow of the Life Sciences Research Foundation. This work was supported by NIH grants to M.T.L. (R01GM082899) and L.A.M. (U54CA143874).

Supplementary Materials

www.sciencemag.org/content/342/6159/731/suppl/DC1
Materials and Methods
Figs. S1 to S34
Tables S1 to S4
References (23–25)

17 June 2013; accepted 8 October 2013
Published online 24 October 2013;
10.1126/science.1242059

Mitochondrial Fusion Directs Cardiomyocyte Differentiation via Calcineurin and Notch Signaling

Atsuko Kasahara,¹ Sara Cipolat,^{2*} Yun Chen,³ Gerald W. Dorn II,^{3†} Luca Scorrano^{2,4†}

Mitochondrial morphology is crucial for tissue homeostasis, but its role in cell differentiation is unclear. We found that mitochondrial fusion was required for proper cardiomyocyte development. Ablation of mitochondrial fusion proteins Mitofusin 1 and 2 in the embryonic mouse heart, or gene-trapping of *Mitofusin 2* or *Optic atrophy 1* in mouse embryonic stem cells (ESCs), arrested mouse heart development and impaired differentiation of ESCs into cardiomyocytes. Gene expression profiling revealed decreased levels of transcription factors transforming growth factor- β /bone morphogenetic protein, serum response factor, GATA4, and myocyte enhancer factor 2, linked to increased Ca^{2+} -dependent calcineurin activity and Notch1 signaling that impaired ESC differentiation. Orchestration of cardiomyocyte differentiation by mitochondrial morphology reveals how mitochondria, Ca^{2+} , and calcineurin interact to regulate Notch1 signaling.

Given their participation in metabolism, signaling, and cell death (1), mitochondria are essential for tissue homeostasis. During development, mitochondria control apoptosis and supply adenosine triphosphate (ATP) essential for differentiation (2), which is especially important in energy-demanding cells like cardiomyocytes

(3). Organellar morphology regulates both mitochondrial apoptosis and ATP production: Ablation of the inner mitochondrial membrane mitochondria-shaping protein optic atrophy 1 (OPA1) (4) and of the outer mitochondrial membrane mitofusin (MFN)–1 and –2 (5) is embryonic lethal, and their conditional deletion in cardiomyocytes causes car-

diomyopathy in *Drosophila* (6) and in the mouse (7, 8). How mitochondria participate in developmental cascades remains unclear.

We investigated embryonic lethality provoked by cardiomyocyte-restricted, combined *Mfn1* and *Mfn2* ablation (KO) (8, 9). Frequencies of KO embryos between embryonic day 8.5 (E8.5) and E10.5 were only slightly depressed, whereas lethality occurred thereafter (Fig. 1A, fig. S1A, and table S1). At E9.5, hearts of control and KO embryos were nearly indistinguishable, but by E12.5 to E13.5, KO hearts were markedly hypoplastic, with biventricular wall thinning and poor trabeculation (Fig. 1B and figs. S1 and S2). Mitochondria of KO cardiomyocytes were fragmented (fig. S3). Steady-state mRNA expression levels of some central embryonic cardiomyocyte differentiation and proliferation regulators were decreased at E9.5 (Fig. 1C and fig. S4). Accord-

¹Department of Cell Physiology and Metabolism, University of Geneva, 1206 Geneva, Switzerland. ²Dulbecco-Telethon Institute, Venetian Institute of Molecular Medicine, 35129 Padova, Italy. ³Department of Internal Medicine, Center for Pharmacogenomics, Washington University School of Medicine, St. Louis, MO, USA. ⁴Department of Biology, University of Padova, 35121 Padova, Italy.

*Present address: Centre for Stem Cells and Regenerative Medicine, King's College London, London SE1 9RT, UK.

†Corresponding author. E-mail: luca.scorrano@unipd.it (L.S.); gdorn@DOM.wustl.edu (G.W.D.)

ingly, only $0.59 \pm 0.12\%$ (SEM, $n = 3$) of myosin heavy-chain (MYH6/7) positive KO cardiomyocytes expressed the proliferation marker phosphorylated histone 3 (pHH3), versus $1.25 \pm 0.34\%$ (SEM, $n = 3$) in control hearts ($P = 0.03$) (fig. S5A), without evidence of apoptosis (fig. S5B).

During in vitro differentiation of embryonic stem cells (ESCs) to cardiomyocytes (10), mitochondrial elongation was accompanied by increased MFN2 (1.91 ± 0.27 fold) and OPA1 (1.67 ± 0.19 fold; SEM, $n = 5$, $P = 0.05$) levels (Fig. 1, D and E). These mitochondrial fusion factors proved essential for cardiomyocyte differentiation: Lowering ESCs MFN2 and OPA1 levels by gene trapping (gt) (fig. S6, A to C) did not affect chromosomal content (fig. S6D), pluripotency (fig. S6E), or mitochondrial mass (fig. S6, B, C, and F), but it impaired differentiation into beating cardiomyocytes (Fig. 1F and fig. S7), which was restored by retroviral re-expression of MFN2 and OPA1 (Fig. 1F and fig. S8). The developmental arrest was specific for the cardiomyocyte lineage

because levels of the mesodermal marker Brachyury were normal in *Mfn2*^{gt}- and *Opal*^{gt}-differentiating ESCs (Fig. 2A), whereas cardiomyocyte-specific *Mef2c2* and *Nkx2.5* mRNA levels were reduced (Fig. 2, B and C, and fig. S9).

Interrupting mitochondrial fusion had the greatest impact on *Mef2c* and GATA4 transcriptional nodes that are downstream of the Notch1 pathway (11). *Notch1* mRNA levels were not significantly altered in KO embryos (fig. S4C) or gt ESCs (fig. S9), but a fluorescent gene reporter assay (12) uncovered Notch1 hyperactivity in *Mfn2*^{gt} and *Opal*^{gt} ESCs (Fig. 2, D and E). Accordingly, nuclear content of the active Notch1 intercellular domain (NICD1) was increased (Fig. 2F), whereas overall NICD1 levels produced by γ -secretase (13) were unchanged (fig. SF10).

Although mitochondria appeared fragmented in undifferentiated and differentiating gene-trapped ESCs (figs. S11 and S12), multiple indices of mitochondrial function and apoptosis were normal (figs. S13 and S14). Conversely, capacitative

Ca^{2+} entry (CCE), which is both modulated by mitochondria and can direct key cardiac transcription pathways (14), was increased in *Mfn2*^{gt} and *Opal*^{gt} ESCs (Fig. 3, A and B), despite that key calcium regulatory proteins were unaffected (fig. S15). The Ca^{2+} -sensitive transcriptional regulator calcineurin A (CnA) (15) was also hyperactivated during differentiation (Fig. 3C). Total internal reflection fluorescence (TIRF) microscopy showed increased mitochondria in proximity to the plasma membranes in *Mfn2*^{gt} and *Opal*^{gt} ESCs (Fig. 3, D and E), potentially sustaining CCE by buffering Ca^{2+} at plasmalemmal CCE channels (16).

Mechanistically, a crosstalk between Ca^{2+} /CnA and Notch1 signaling was revealed by the Notch1 activity augmentation induced by constitutively active CnA (17) or by ionomycin, a CnA-activating Ca^{2+} ionophore (14) (Fig. 4, A and B). CnA inhibition by FK506, but not NFAT inhibition by VIVIT (18), prevented Notch1 stimulation by ionomycin (Fig. 4A and fig. S16). Likewise, ionomycin did not increase NICD1 production (fig. S17), but it induced

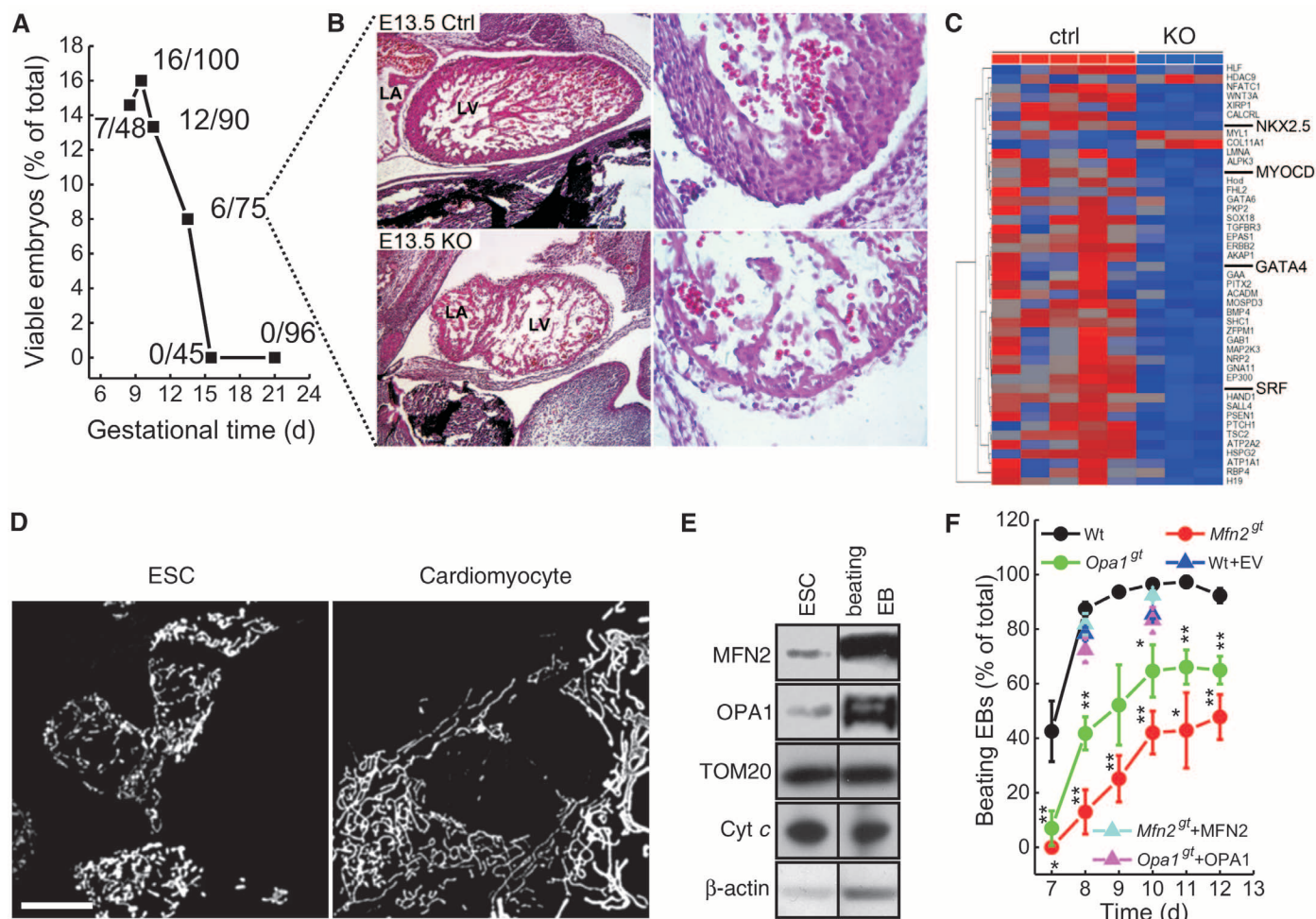


Fig. 1. Mitochondrial fusion is required for myocardial development and cardiomyocyte differentiation. (A) Viability of mouse embryos with cardiomyocyte-specific *Mfn1* and *Mfn2* ablation. Numbers of double-knockout (KO) embryos genotyped are indicated. (B) Hematoxylin-eosin staining of E13.5 Ctrl and KO hearts in sagittal section. Note the ventricular wall thinning. (C) Heat map of RNA-sequence analysis of cardiac gene expression in control (CTRL) and KO E9.5 embryos.

(D) Three-dimensional reconstructions of z stacks of confocal mitochondrial yellow fluorescent protein images in the indicated cells. Scale bar, 10 μm . (E) Immunoblots using the indicated antibodies of proteins (30 μg) isolated from the indicated stage of differentiation. (F) Impaired differentiation of *Mfn2*^{gt} and *Opal*^{gt} ESCs into beating embryoid bodies (EBs). Where indicated, cells were transduced with the indicated retrovirus. Mean $\pm$ SEM ($N = 8$, $*P < 0.05$, $**P < 0.005$ versus wild type (wt)).

Fig. 2. Ablation of mitochondrial fusion increases Notch activity. (A) Immunoblots using the indicated antibodies of proteins (30 μ g) from the indicated differentiating ESCs. (B and C) Real-time polymerase chain reaction of *Mef2c2* (B) and *Nkx2.5* (C) mRNA in the indicated differentiating ESCs. Mean $\pm$ SEM ($N = 3$, $*P < 0.05$ versus wt). (D) Fluorescence images of Notch1 activity reporter during differentiation of the indicated ESCs. Scale bar, 50 μ m. (E) Mean $\pm$ SEM of experiments as in (D) ($N = 4$, $*P < 0.05$ versus wt). (F) (Top) Immunoblots using the indicated antibodies of nuclear pellet proteins (15 μ g) from the indicated ESCs. (Bottom) Mean $\pm$ SEM of densitometric data ($N = 4$, $*P < 0.05$ versus wt).

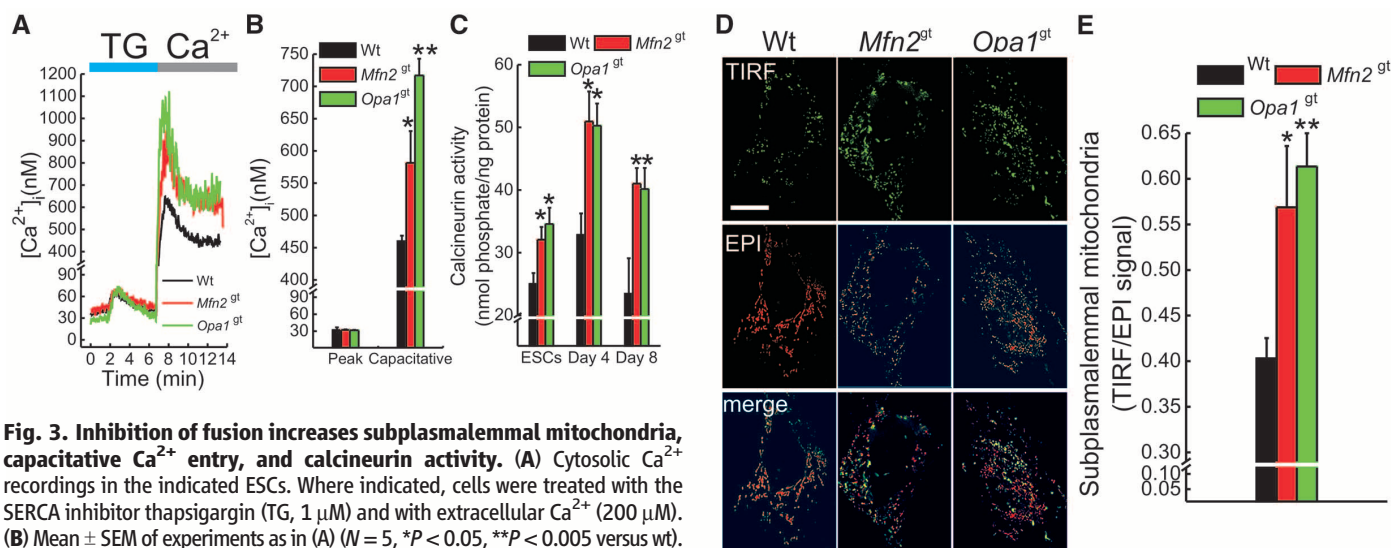
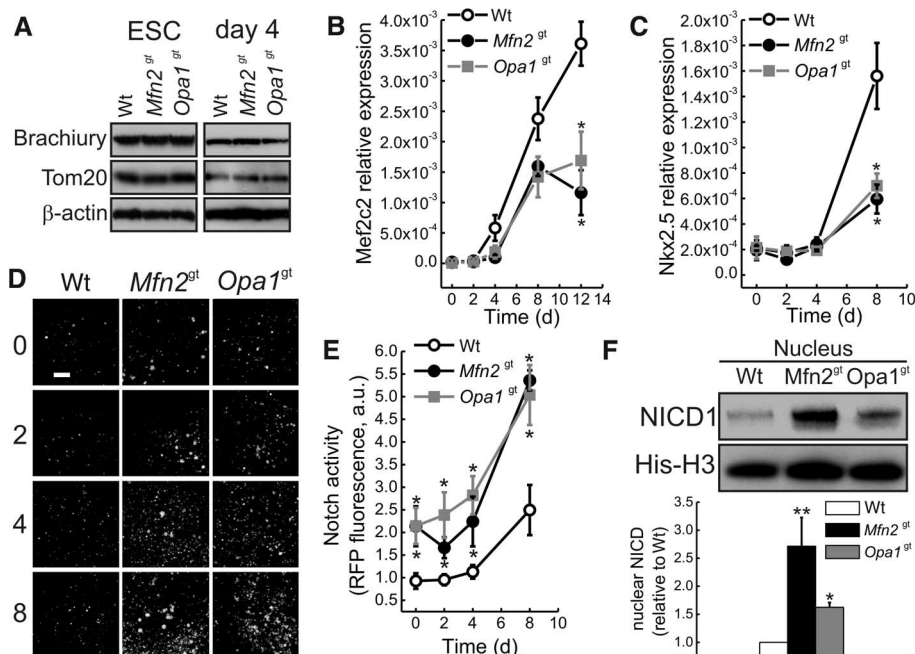


Fig. 3. Inhibition of fusion increases subplasmalemmal mitochondria, capacitive Ca^{2+} entry, and calcineurin activity. (A) Cytosolic Ca^{2+} recordings in the indicated ESCs. Where indicated, cells were treated with the SERCA inhibitor thapsigargin (TG, 1 μ M) and with extracellular Ca^{2+} (200 μ M). (B) Mean $\pm$ SEM of experiments as in (A) ($N = 5$, $*P < 0.05$, $**P < 0.005$ versus wt). (C) Mean $\pm$ SEM of cytoplasmic calcineurin activity in the indicated differentiating ESCs ($N = 3$, $*P < 0.05$ versus wt). (D) Subplasmalemmal (TIRF, green) and total (epifluorescence, EPI, red) fluorescence of mitochondrial *Drosophila* sp. red fluorescent protein (dsRED) acquired by TIRF microscopy in the indicated ESCs. Scale bar, 10 μ m. (E) Mean $\pm$ SEM of experiments as in (D) ($N = 4$, $*P < 0.05$; $**P < 0.005$ versus wt).

FK506-sensitive NICD1 nuclear accumulation (Fig. 4B).

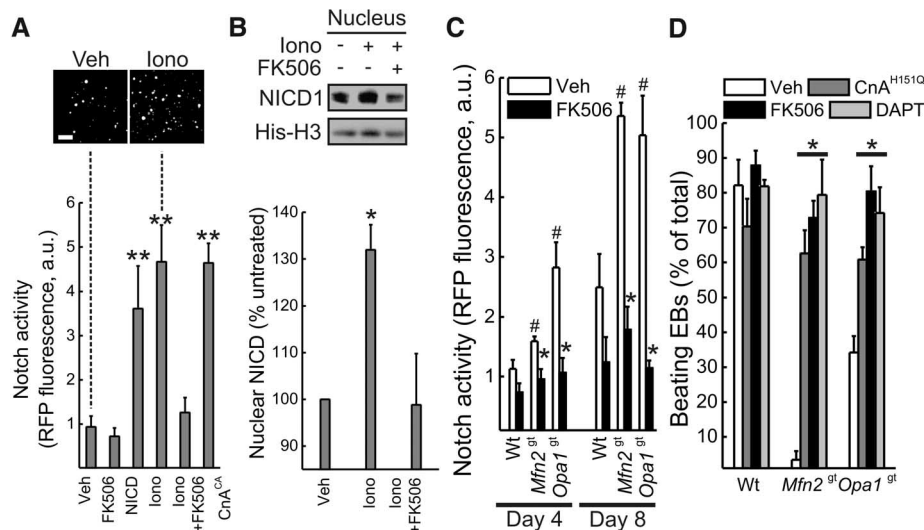
Finally, we demonstrated that impaired cardiomyocyte differentiation provoked by interrupting mitochondrial fusion requires stimulation of CnA-mediated Notch signaling. We observed that constitutive CnA activation was sufficient to block ESCs differentiation and that this differentiation inhibition was partially reversed by the NICD production inhibitor DAPT (fig. S18). Likewise, genetic and pharmacological CnA inhibition normalized Notch activity (Fig. 4C and fig. S19) and overcame the cardiomyocyte differentiation block in *Mfn2^{gt}* and *Opa1^{gt}* ESCs (Fig. 4D), without normalizing mitochondrial morphometry (fig. S20) or subplasmalemmal

mitochondrial redistribution (fig. S21). Thus, as regulators of cardiomyocyte differentiation, CnA activity and downstream Notch1 signaling are centrally directed by mitochondrial dynamics.

We demonstrate a critical crosstalk between Ca^{2+} /calcineurin and Notch1 signaling cascades during cardiomyocyte differentiation, uncovering an additional signaling pathway that may function in addition to, or in parallel with, Notch1 regulation by extracellular Ca^{2+} (19) and the sarcoplasmic/endoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA) (20). This advance in our understanding of Notch1 regulation opens the possibility that Notch and calcineurin signaling pathways may also be functionally linked in other tissues.

Mitochondrial shape change is a recognized player in apoptosis. Our results implicate mitochondrial dynamics in cardiomyocyte differentiation as well. Mitochondrial fusion impinges on Ca^{2+} and CnA to control Notch1 inhibition of the mesoderm to cardiomyocyte transition (11) (fig. S22). Mitochondrial morphology, therefore, regulates cardiomyocyte differentiation/proliferation and embryonic heart development, in addition to known modulatory effects on *Drosophila melanogaster* follicle cells (21) and placental syncytiotrophoblast differentiation (22). Notably, impaired cardiomyocyte differentiation in mitochondrial fusion-defective ESCs was not a consequence of altered ATP availability, as described in other contexts (23). Instead,

Fig. 4. Calcineurin inhibition corrects Notch1 activity and cardiomyocyte differentiation of fusion-deficient ESCs. (A) (Top) Fluorescence images of Notch1 activity reporter in ESCs treated as indicated. Ionomycin (iono, 1 μ M), Ca^{2+} ionophore; Veh, vehicle. Scale bar, 50 μ m. (Bottom) Mean $\pm$ SEM of Notch activity in the indicated ESCs transfected or treated as indicated. CnA^{CA} , constitutively active CnA ($N = 6$, $**P < 0.005$ versus Veh.). (B) (Top) Immunoblots using the indicated antibodies of nuclear pellet proteins (15 μ g) from ESCs treated as indicated. (Bottom) Mean $\pm$ SEM of densitometric data ($N = 3$, $*P < 0.05$ versus Veh.). (C) Mean $\pm$ SEM of Notch1 activity in the indicated differentiating ESCs treated as indicated ($N = 4$, $*P < 0.05$ versus Veh., $\#P < 0.05$ versus wt). (D) Mean $\pm$ SEM of beating EBs from the indicated ESCs transfected or treated as indicated. $\text{CnA}^{\text{H151Q}}$, dominant-negative CnA mutant; DAPT, NICD production inhibitor ($N = 4$, $**P < 0.005$ versus wt).



our results uncover a mitochondrial fusion-sensitive Ca^{2+} /calcineurin pathway that regulates the central cardiac development factor Notch1, interrupting cardiomyocyte proliferation and blocking fetal cardiac development.

Cell fate in the developing fetus is determined by key nuclear transcription factors that drive specific differentiation gene programs. Mitochondria, the descendant of primordial bacteria living within unicellular organisms, participate in these processes by fueling them and by determining apoptotic and nonapoptotic cell death. Our findings invert this paradigm, demonstrating how a primary disturbance in organelle morphology can redirect the developmental fate of the host cell.

References and Notes

1. D. R. Green, G. Kroemer, *Science* **305**, 626–629 (2004).
2. S. Mandal, A. G. Lindgren, A. S. Srivastava, A. T. Clark, U. Banerjee, *Stem Cells* **29**, 486–495 (2011).
3. S. Chung et al., *Nat. Clin. Pract. Cardiovasc. Med.* **4** (suppl. 1), S60–S67 (2007).
4. M. V. Alavi et al., *Brain* **130**, 1029–1042 (2007).
5. H. Chen, J. M. McCaffery, D. C. Chan, *Cell* **130**, 548–562 (2007).
6. G. W. Dorn 2nd et al., *Circ. Res.* **108**, 12–17 (2011).
7. Y. Chen, Y. Liu, G. W. Dorn 2nd, *Circ. Res.* **109**, 1327–1331 (2011).
8. Y. Chen, G. W. Dorn 2nd, *Science* **340**, 471–475 (2013).
9. Detailed experimental procedures are in the supplementary materials on Science Online.
10. G. M. Keller, *Curr. Opin. Cell Biol.* **7**, 862–869 (1995).
11. H. Shen et al., *Genes Dev.* **20**, 675–688 (2006).
12. M. Nemir, A. Croquelois, T. Pedrazzini, F. Radtke, *Circ. Res.* **98**, 1471–1478 (2006).
13. B. De Strooper et al., *Nature* **398**, 518–522 (1999).
14. R. E. Dolmetsch, R. S. Lewis, C. C. Goodnow, J. I. Healy, *Nature* **386**, 855–858 (1997).
15. V. Costa et al., *EMBO Mol. Med.* **2**, 490–503 (2010).
16. M. Hoth, C. M. Fanger, R. S. Lewis, *J. Cell Biol.* **137**, 633–648 (1997).
17. G. M. Cereghetti et al., *Proc. Natl. Acad. Sci. U.S.A.* **105**, 15803–15808 (2008).
18. J. D. Molkentin et al., *Cell* **93**, 215–228 (1998).
19. M. D. Rand et al., *Mol. Cell. Biol.* **20**, 1825–1835 (2000).
20. G. Roti et al., *Cancer Cell* **23**, 390–405 (2013).

21. K. Mitra, R. Rikhy, M. Lilly, J. Lippincott-Schwartz, *J. Cell Biol.* **197**, 487–497 (2012).
22. M. Wasilewski et al., *Curr. Biol.* **22**, 1228–1234 (2012).

Acknowledgments: We thank F. Radtke and R. Kopan for plasmids, B. Wehrle-Haller for help with TIRF imaging, and D. Martinvalet and N. Demareux for helpful discussions. L.S. is a Dulbecco Telethon Institute Senior Scientist. A.K. received the ISHR-ES/SERVIER Research Fellowship, and S.C. received an Associazione Italiana Ricerca sul Cancro (AIRC) Fellowship. This work was supported by Telethon GGP06254A, GPP10005B; AIRC; SNF 31-118171; Oncosuisse; ERC StG 282280 (to L.S.); and NIH R01 HL59888 (to G.W.D.). The data presented in this paper are tabulated in the main paper and the supplementary materials.

Supplementary Materials

www.sciencemag.org/content/342/6159/734/suppl/DC1
Materials and Methods
Figs. S1 to S22
Table S1
References (23–28)

3 June 2013; accepted 24 September 2013
Published online 3 October 2013;
10.1126/science.1241359

The Hippo Signaling Pathway Interactome

Young Kwon,¹ Arunachalam Vinayagam,^{1*} Xiaoyun Sun,^{3*} Noah Dephore,⁴ Steven P. Gygi,⁴ Pengyu Hong,³ Norbert Perrimon^{1,2†}

The Hippo pathway controls metazoan organ growth by regulating cell proliferation and apoptosis. Many components have been identified, but our knowledge of the composition and structure of this pathway is still incomplete. Using existing pathway components as baits, we generated by mass spectrometry a high-confidence *Drosophila* Hippo protein-protein interaction network (Hippo-PPIN) consisting of 153 proteins and 204 interactions. Depletion of 67% of the proteins by RNA interference regulated the transcriptional coactivator Yorkie (Yki) either positively or negatively. We selected for further characterization a new member of the alpha-arrestin family, Leash, and show that it promotes degradation of Yki through the lysosomal pathway. Given the importance of the Hippo pathway in tumor development, the Hippo-PPIN will contribute to our understanding of this network in both normal growth and cancer.

Central to the Hippo pathway are the two protein kinases Hippo and Warts (Wts) kinases that regulate the Yki transcriptional coactivator (1–3). Yki together with the TEA/AATS

domain (TEAD) transcription factor Scalloped induces the transcription of a number of targets that promote cell proliferation and survival. Hippo activates Wts by phosphorylation, which in turn

inhibits Yki by phosphorylation, causing its cytoplasmic retention by association with 14-3-3. Hippo signaling is regulated by the protocadherins Fat (Ft) and Dachsous (Ds) (1–3). Ft regulates Wts independently of Hpo by affecting the stability of Wts through the unconventional myosin Dachs (D) (4). A number of other proteins, including Merlin (Mer), Kibra, Tao, Salt-inducible kinases, and cell polarity proteins, also regulate the core kinases (5, 6).

Most components of the Hippo pathway have been identified from genetic screens in *Drosophila*. To complement these approaches and gain information on the organization of the signaling

¹Department of Genetics, Harvard Medical School, Boston, MA 02115, USA. ²Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA. ³Department of Computer Science, Volen Center for Complex Systems, Brandeis University, Waltham, MA 02454, USA. ⁴Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: perrimon@receptor.med.harvard.edu

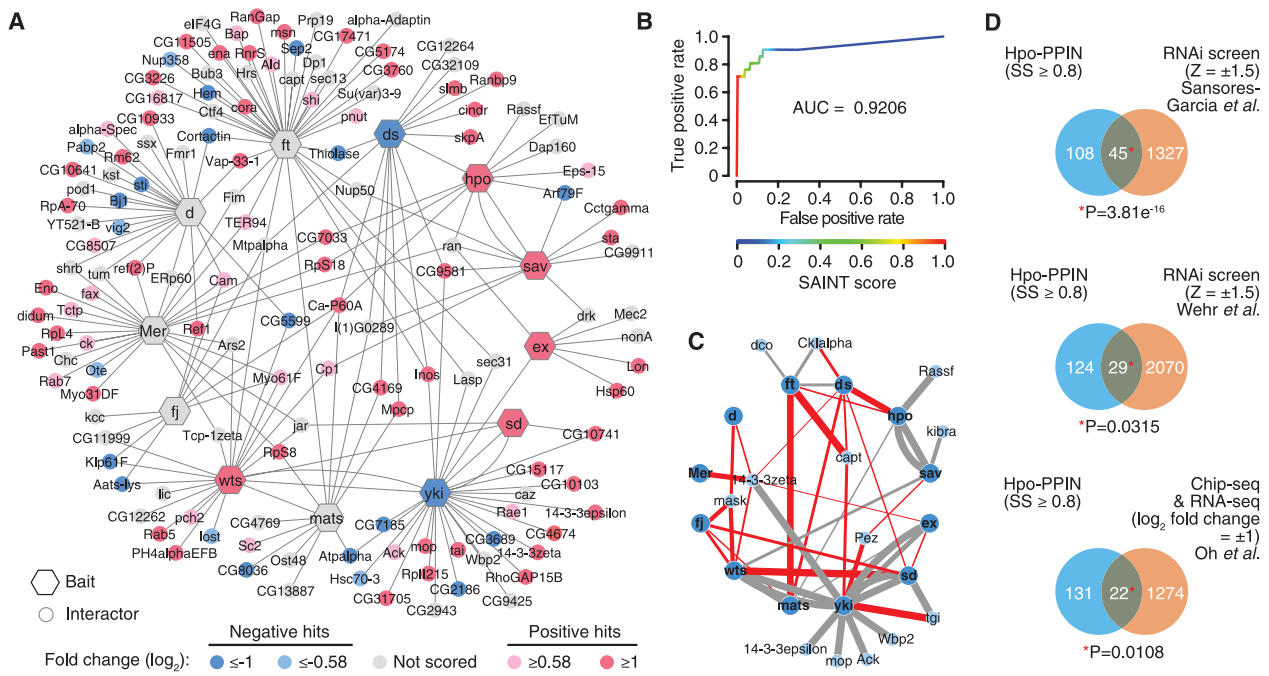
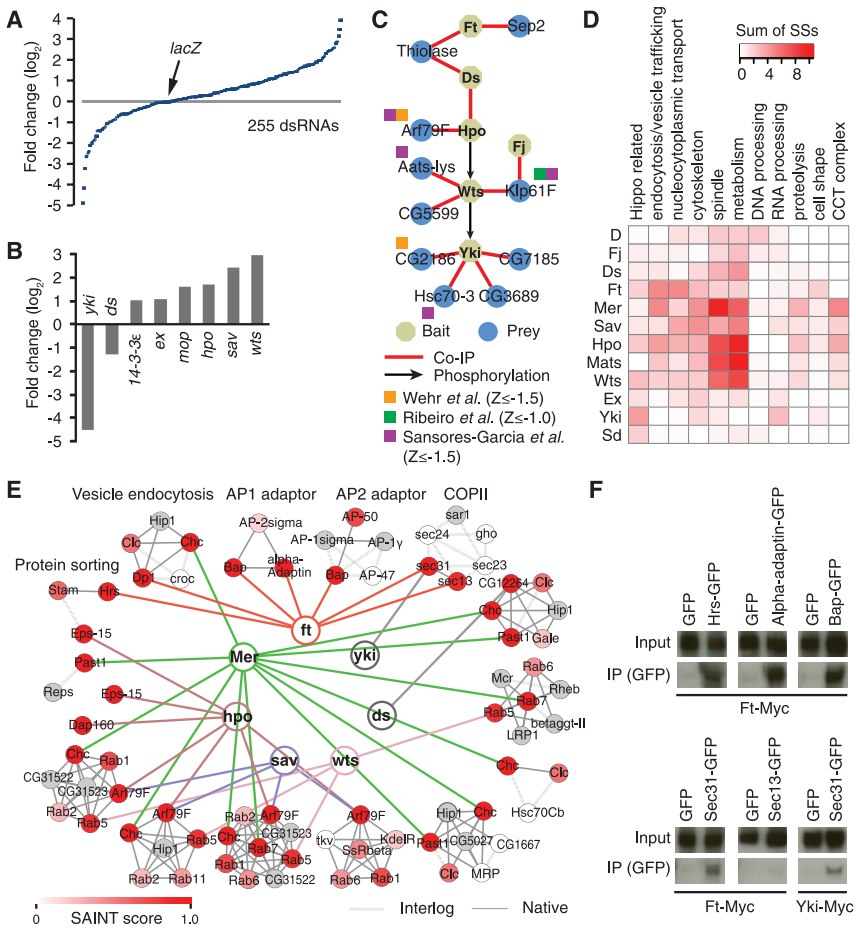


Fig. 1. Proteomic identification of PPIs surrounding the Hippo pathway. (A) Network representation of the functional Hippo-PPIN. PPIs with SS ≥ 0.8 are shown. Node color indicates the RNAi screen results. (B) Receiver operating characteristic (ROC) curve showing the performance of SAINT score. The true-positive rate and false-positive rate are computed at various SAINT score cutoffs. The area under curve (AUC) is 0.9206. (C) Recovered PPIs

among known components from the PPIN. Gray edges indicate the known interactions recapitulated from the PPIN. Red edges indicate novel interactions recovered from the PPIN. Edge thickness corresponds to SS. (D) Comparison of Hippo-PPIN with the published RNAi screen, Chip-seq, and RNA-seq data sets. Yki targets correspond to the genes identified by both Yki-Chip and *wts*^{P2}-RNA sequencing data.

Fig. 2. Validation of Hippo-PPIN with functional RNAi screen and co-IP. (A) Distribution of Yki-reporter values for individual double-stranded RNAs (dsRNAs) in our focused RNAi screen. About 70% of genes are covered by two dsRNAs. (B) Recovery of Hippo pathway components from RNAi screen [fold-change (log₂) cutoff ± 1]. (C) The positive regulatory network validated with co-IP. (D) Heat map, based on COMPLEAT analysis (fig. S5 and table S7), showing interaction between baits (left side) and selected cellular processes (top). The sum of SSs for the PPIs between bait and subcomplexes in a biological process is used to show the interaction strength. (E) Interaction network with subcomplexes involved in endocytosis and vesicle trafficking. All edges with baits have SS ≥ 0.8. Node color represents the highest SS. (F) Co-IP validation of novel PPIs (SS ≥ 0.8) with subcomplexes related to endocytosis and vesicle trafficking.



network, we constructed a PPIN centered on the *Drosophila* Hippo canonical pathway components by affinity purification and mass spectrometry (AP/MS) from *Drosophila* S2R⁺ cells (7, 8). In total, we identified an unfiltered network of 4560 interactions (edges) among 1518 proteins (nodes). The total spectral counts for each identified protein were processed using the Significance Analysis of Interactome (SAINT) algorithm that confers a confidence score for PPI data obtained with AP/MS (9). Evaluation of the network by SAINT score (SS) performance test reveals that it is of high quality (Fig. 1B). PPIs with SS ≥ 0.8 (false positive rate $< 3\%$; 204 interactions and 153 proteins) are shown in Fig. 1A (see table S1 for the full list of interactions and table S2 for the list of human orthologs of proteins with SS ≥ 0.8). We also compared the Hippo-PPIN with literature-based physical and genetic interaction networks (7). The overlap between Hippo-PPIN and the literature-based networks increased along with the increase of SS (fig. S1 and table S3), indicating that SS reliably represents the confidence of PPIs. Furthermore, we experimentally validated 23 out of 26 PPIs ($\sim 13\%$ of the high-confidence PPIs; SS ≥ 0.8) with coimmunoprecipitation (co-IP) assay (fig. S2), leading to an estimate of $\sim 11.5\%$ experimental false positive rate with SS ≥ 0.8 .

Of known biochemical interactions between Hippo pathway components, 16 out of 31 expected interactions (table S4) with the baits ($\sim 52\%$

coverage) were represented in the Hippo-PPIN (Fig. 1C, gray edges). Importantly, our Hippo-PPIN uncovered a number of new interactions between known components, including Ft-Capulet (Capt), Ft-Mob as tumor suppressor (Mats), Ds-Hippo, and Yki-Tondu-domain-containing growth inhibitor (Tgi) (Fig. 1C, red edges). Moreover, the Hippo-PPIN was significantly enriched for the hits from the published RNA interference (RNAi) screens (5, 10) and the potential transcriptional targets of Yki (11) (Fig. 1D and fig. S3).

To address the contribution of each node to the regulation of the downstream transcription factor Yki, we performed a focused-RNAi screen for 98% of the high-confidence nodes (SS ≥ 0.8) (see supplementary materials) (Fig. 2A). This screen efficiently recovered multiple known pathway components (Fig. 2B) and provided a functional validation for 67% of the high-confidence nodes, comprising 77 negative and 25 positive regulators with fold-change ($\log_2$) cutoff ± 0.58 (see supplementary materials) (Fig. 1A and table S5).

Existing Hippo pathway components are enriched for negative effectors of Yki because their overgrowth phenotypes and increase of *yki*-reporter activity in vivo are readily detectable. By combining proteomics and functional genomics data, we identified 19 positive effectors of *yki* with fold-change ($\log_2$) ≤ -1 . We tested 15 PPIs with the positive effectors based on the availability of cDNA clones and validated 13 PPIs with co-IP (Fig. 2C and fig. S4). Additional-

ly, positive effectors such as Arf79F, CG2186, Aats-lys, Hsc70-3, and Klp61F were independently identified in (5, 10, 12) (Fig. 2C).

To gain further insights on the organization of Hippo-PPIN, we used the Protein Complex Enrichment Analysis Tool (COMPLEAT) (13), which identifies enriched known protein complex in a data set based on a comprehensive protein complex resource. The confidence of the interaction between a complex and Hippo pathway components over the entire unfiltered PPIN was represented with interquartile means of SSs for nodes in a complex (Complex Score). From this analysis, we identified 299 subcomplexes from the entire network (table S6) that revealed potential links with multiple biological processes impinging on Hippo signaling (Fig. 2D, fig. S5, and table S7). Notably, consistent with the observation that multiple actin regulators affect Hippo signaling (10, 14), interactions with cytoskeletal complexes were prominent (fig. S6). Further, reminiscent of the function of a number of Hippo pathway components in the regulation of cell division orientation (15) and positioning anaphase spindle and astral microtubules (16), many complexes related to spindle organization were identified (fig. S6). Strong association with endocytosis and vesicle trafficking complexes was also uncovered (Fig. 2E). Among the high-confidence PPIs, we validated 5 PPIs between Ft and endocytic components, and PPIs of Yki-Sec31 and Hpo-Arf79F (Fig. 2F and fig. S4). Furthermore, our RNAi screen validated a few high-confidence nodes as positive and negative effectors of Yki (fig. S6E). Given the active involvement of intracellular vesicles in multiple signaling events, our results implicate potential roles for vesicle trafficking in various aspects of Ft and Hippo signaling.

The Hippo-PPIN contains many potential new components of Hippo signaling. To initiate their characterization, we focused on an interactor of Yki, CG4674, which belongs to the arrestin domain containing (Ardc) protein family. CG4674 is of particular interest because its mammalian orthologs, including Ardc3, are implicated in tumor suppression by regulating cell proliferation and survival (17).

Co-IP (Fig. 3A) and reciprocal AP/MS (table S8) further established the association between CG4674 and Yki. Depletion of CG4674 by RNAi increased Yki-reporter activity (Fig. 3B), and overexpression had the opposite effect (Fig. 3C), suggesting that CG4674 restrains Yki activity (thus leading us to refer to CG4674 as *leash*). Although Yki was found in the cytoplasm, nucleus, and cytoplasmic vesicles, *Leash* predominantly localized to cytoplasmic vesicles. However, when Yki and *Leash* were expressed together, strong colocalization was observed (Fig. 3D). Importantly, overexpression and RNAi of *leash* increased and decreased vesicular localization of Yki, respectively (Fig. 3, E and F). Further, the late endosome marker, Rab9, colocalized with a subset of Yki-positive vesicles, and the lysosomal marker, Lamp1, encircled some Yki-positive punctae (fig. S7).

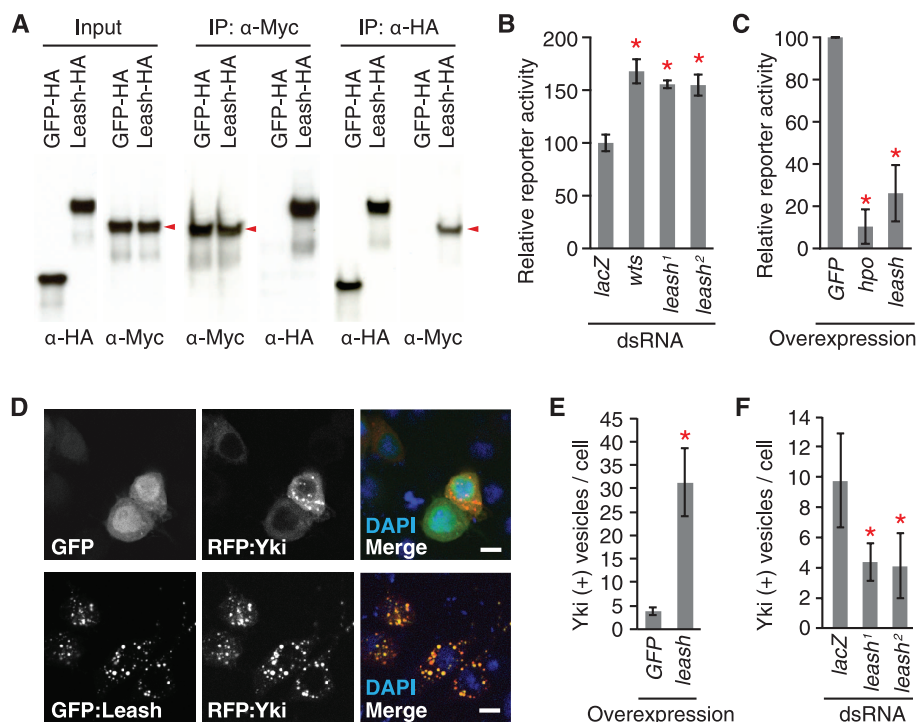


Fig. 3. Identification of *Leash* as a regulator of Yki. (A) co-IP. Arrowheads indicate Yki-Myc. (B) and (C) Yki-reporter assay with knockdown (B) or overexpression (C) of *leash*. Nonoverlapping dsRNAs against *leash* (1, DRSC15617; 2, DRSC28712) were used. (D) Induction of vesicular localization of Yki by *Leash*. (E) Quantification of Yki-containing vesicles following *leash* expression. (F) Quantification of Yki-containing vesicles after *leash* knockdown. Mean $\pm$ SDs are shown. * $P \leq 0.05$, Student's *t* test.

Overexpression of *wts* significantly increased the abundance of Yki-containing vesicles (fig. S8), suggesting that Hippo signaling regulates the vesicular localization of Yki. Arrdc proteins have conserved arrestin domains at their N-termini that interact with their cargos. Expression of Leash N-terminal arrestin domains alone was sufficient to regulate Yki activity by forming a complex (fig. S9), indicating that Yki is a Leash cargo.

We also identified additional Leash interactors by AP/MS (tables S8 and S9) and found that Leash strongly binds to Nedd4 and a few other HECT (homologous to E6-AP carboxyl terminus) ubiquitin ligases (Fig. 4A). Moreover, we identified three ubiquitinated lysines on Leash that were important for complex formation with Yki (fig. S10). To address whether human Arrdcs could down-regulate Yki, we tested five human Leash orthologs (fig. S11). Expression of Arrdc1 or Arrdc3 reduced Yki-reporter activity (fig. S11B) and Yki protein

abundance (Fig. 4B). Because Nedd4 induces degradation of its substrate through the endosomal-lysosomal pathway (18), we tested the effect of Bafilomycin, an inhibitor of lysosome acidification. Bafilomycin treatment increased Yki activity (Fig. 4C) and reversed the effect of overexpression of full-length Leash or Arrestin domains (Fig. 4D), indicating that Leash decreases Yki abundance through the lysosomal degradation pathway.

Yki activity is a critical determinant of growth, and Hippo signaling restricts growth by suppressing Yki. Consistent with the role of Leash in inhibiting Yki, overexpression of *leash* or *arrdc3* reduced wing size significantly (Fig. 4E and fig. S12). Further, depletion of *leash* in wing discs affected neither wing size nor Yki protein abundance significantly, suggesting functional redundancy between family members or additional regulatory mechanisms. In the midgut, under normal homeostasis, Yki is inhibited. However, when

an active form of *yki* (*yki*^{3S/A}) is expressed or an upstream regulator is inactivated, midgut stem cells overproliferate (19, 20). Depletion of *leash* enhanced proliferation induced by *hippo* knockdown without affecting proliferation under normal homeostasis (Fig. 4F). Furthermore, overexpression of *leash* or *arrdc3* suppressed *yki*^{3S/A}-induced proliferation (Fig. 4G). Altogether, these results indicate that *leash* is a negative regulator of Yki.

In summary, we generated a PPIN for the Hippo pathway. Analysis of the interactions using functional RNAi screen and COMPLEAT revealed a snapshot of the overall organization of the Hippo signaling network. Further, we characterized Leash, a novel component of the Hippo pathway, and showed that it down-regulates Yki through lysosomal degradation. Altogether, the Hippo-PPIN provides a resource for further in-depth characterization of new and existing components of the Hippo pathway.

References and Notes

1. D. Pan, *Dev. Cell* **19**, 491–505 (2010).
2. B. K. Staley, K. D. Irvine, *Dev. Dyn.* **241**, 3–15 (2012).
3. G. Halder, R. L. Johnson, *Development* **138**, 9–22 (2011).
4. E. Cho *et al.*, *Nat. Genet.* **38**, 1142–1150 (2006).
5. M. C. Wehr *et al.*, *Nat. Cell Biol.* **15**, 61–71 (2013).
6. F. X. Yu, K. L. Guan, *Genes Dev.* **27**, 355–371 (2013).
7. A. A. Friedman *et al.*, *Sci. Signal.* **4**, rs10 (2011).
8. P. Kyriakakis, M. Tipping, L. Abed, A. Veraksa, *Fly (Austin)* **2**, 229–235 (2008).
9. H. Choi *et al.*, *Nat. Methods* **8**, 70–73 (2011).
10. L. Sansores-Garcia *et al.*, *EMBO J.* **30**, 2325–2335 (2011).
11. H. Oh *et al.*, *Cell Rep* **3**, 309–318 (2013).
12. P. S. Ribeiro *et al.*, *Mol. Cell* **39**, 521–534 (2010).
13. A. Vinayagam *et al.*, *Sci. Signal.* **6**, rs5 (2013).
14. G. Halder, S. Dupont, S. Piccolo, *Nat. Rev. Mol. Cell Biol.* **13**, 591–600 (2012).
15. Y. Mao *et al.*, *Genes Dev.* **25**, 131–136 (2011).
16. J. M. Rock *et al.*, *Science* **340**, 871–875 (2013).
17. K. M. Draheim *et al.*, *Oncogene* **29**, 5032–5047 (2010).
18. G. K. Tofaris *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 17004–17009 (2011).
19. P. Karpowicz, J. Perez, N. Perrimon, *Development* **137**, 4135–4145 (2010).
20. F. Ren *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 21064–21069 (2010).

Acknowledgments: We thank the Transgenic RNAi Project, *Drosophila* RNAi Screening Center, and Bloomington Stock Center for fly stocks and reagents; C. Villata, E. Heffern, and R. Binari for technical support; L. Sansores-Garcia and G. Halder for help with the Yki-reporter assay; S. Blair and K. Irvine for reagents; and P. Karpowicz and R. Sopko for comments. Y.K. was supported by the Damon Runyon Cancer Research Foundation. This work was supported by R01-DK088718 and P01-CA120964 to N.P. N.P. is an Investigator of the Howard Hughes Medical Institute. AP/MS data are presented in the supplementary materials. RNAi screen data are available at *Drosophila* RNAi Screening Center.

Supplementary Materials

www.sciencemag.org/content/342/6159/737/suppl/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 to S9
References (21–25)

30 July 2013; accepted 2 October 2013
Published online 10 October 2013;
10.1126/science.1243971

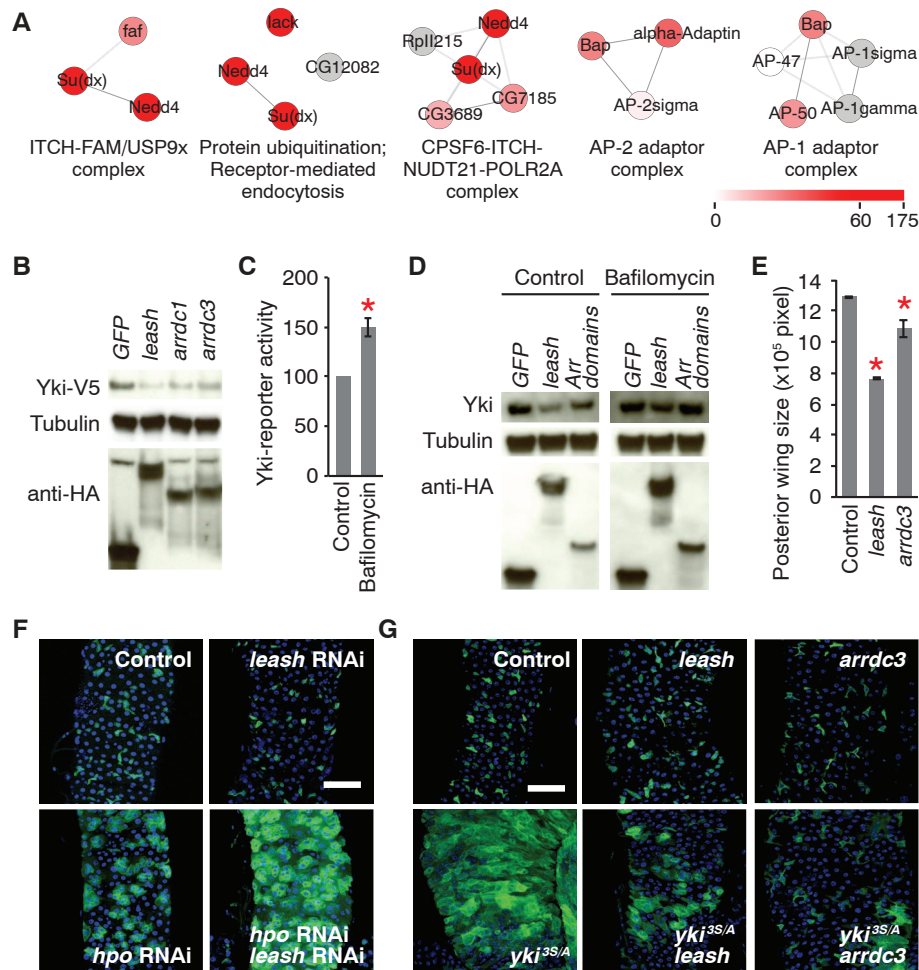


Fig. 4. Mechanism of Yki inhibition by Leash. (A) Subcomplexes identified from the Leash-interactome. Legend shows the sum of spectral counts in triplicates. (B) Yki protein level. (C) Yki-reporter activity. Bafilomycin (0.01 μ M) was treated for 2 days. (D) Suppression of Leash-induced Yki degradation after Bafilomycin treatment. “Arr domains” corresponds to amino acids 1 to 305 of Leash (fig. S9A). (E) Quantification of wing size. (F) Enhancement of *Hippo* knockdown phenotype by *leash* knockdown in gut stem cells. Stem cells and enteroblasts are marked with green fluorescent protein (GFP). Transgenes were induced for 8 days. (G) Suppression of *yki*^{3S/A}-induced proliferation by overexpression of *leash* or *arrdc3*. Transgenes were induced for 6 days. Mean $\pm$ SDs are shown. * $P \leq 0.05$, Student’s *t* test.

High-Speed Force Spectroscopy Unfolds Titin at the Velocity of Molecular Dynamics Simulations

Felix Rico,¹ Laura Gonzalez,² Ignacio Casuso,¹ Manel Puig-Vidal,² Simon Scheuring^{1*}

The mechanical unfolding of the muscle protein titin by atomic force microscopy was a landmark in our understanding of single-biomolecule mechanics. Molecular dynamics simulations offered atomic-level descriptions of the forced unfolding. However, experiment and simulation could not be directly compared because they differed in pulling velocity by orders of magnitude. We have developed high-speed force spectroscopy to unfold titin at velocities reached by simulation (~4 millimeters per second). We found that a small β -strand pair of an immunoglobulin domain dynamically unfolds and refolds, buffering pulling forces up to ~100 piconewtons. The distance to the unfolding transition barrier is larger than previously estimated but is in better agreement with atomistic predictions. The ability to directly compare experiment and simulation is likely to be important in studies of biomechanical processes.

Titin, a molecular spring in muscle sarcomeres, is important in striated muscle function and has been implicated in diseases such as heart failure (1). Titin consists of ~300 modules including immunoglobulin (Ig)-type, fibronectin III-type, and Pro-Glu-Val-Lys (PEVK) domains (2). Force spectroscopy (FS) unfolding of individual titin molecules, using optical tweezers (3, 4) and atomic force microscopy (AFM) (5), opened a new research field relating protein mechanics, structure, and folding. AFM force-extension curves revealed sawtooth-like patterns (periodicity 25 to 28 nm) that indicated unfolding of individual Ig-like domains (5). Combinations of AFM experiments with steered molecular dynamics (SMD) simulations enriched atomic-level descriptions (6–8) of receptor-ligand binding (9, 10) and forced protein unfolding (5). Forced unfolding (pulling speed 0.3 to 0.5 $\mu\text{m/s}$) of titin I91 concatemers (8, 11, 12) resulted in extension of each domain by ~0.7 nm, which correlated with the separation of antiparallel β strands A and B observed in SMD simulations (8, 11). Subsequent rupture of the A'-G β -strand pair led to complete domain unfolding (11, 13, 14). However, a velocity difference of about six orders of magnitude prevented direct comparison of SMD with FS. Indeed, simulations resulted in unfolding forces of ~1 nN, nearly an order of magnitude greater than experimental values (11, 12). Improved computational abilities have allowed simulations that unfolded I91 at 2800 $\mu\text{m/s}$ (still faster than experiment by ~2.5 orders of magnitude), reporting forces of ~500 pN (15).

High-speed AFM [HS-AFM (16)] allows imaging biomolecules at video rate (17–19) through miniaturization of piezoelectric elements and the cantilever (20). We developed an analogous

technique, high-speed FS (HS-FS), with short cantilevers (21) that allowed titin molecules to be pulled at speeds up to ~4000 $\mu\text{m/s}$. This is faster than conventional AFM by ~2.5 orders of magnitude and reaches current limits for SMD simulations.

Our HS-FS setup consists of a miniature piezoelectric actuator and a short cantilever with small viscous damping (0.035 $\text{pN } \mu\text{m}^{-1} \text{ s}^{-1}$) (Fig. 1A and figs. S1 and S2). Titin I91 concatemers were unfolded by HS-FS at pulling velocities ranging over six orders of magnitude, from 0.0097 to 3870 $\mu\text{m/s}$. Only force curves with at

least three sawtooth-like unfolding peaks were analyzed (22) (Fig. 1B and fig. S3). As reported in (5, 23), unfolding forces increased with pulling velocity (Fig. 2A). At slow velocities, HS-FS unfolding forces were in excellent agreement with conventional FS (Fig. 2B). At pulling velocities higher than previously (>100 $\mu\text{m/s}$), unfolding forces followed a steeper slope that reached values greater than 500 pN and overlapped those obtained by simulations (Fig. 2B). Variations in the slope of the plot of mean rupture forces versus the logarithm of the velocity (force spectrum) have been observed for receptor-ligand interactions (24–26) but have rarely been documented for protein unfolding (27), probably because of the limited range of accessible pulling rates.

The microscopic model developed by Hummer and Szabo (26, 28) allowed us to fit the wide range of pulling velocities and describes well the nonlinear upturn in the dynamic force spectrum (26, 28) (Fig. 2B and supplementary materials). According to this theory, at moderate velocities, unfolding is dominated by the pulling rate and stochastic fluctuations (i.e., spontaneous unfolding of the domain under a given force). At high velocities, stochastic fluctuations of the protein along the unfolding pathway become irrelevant and the unfolding process becomes deterministic (28), because the protein is pulled so fast that it has no time to explore its energy landscape.

The slope in the dynamic force spectrum is related to the position of the energy barrier;

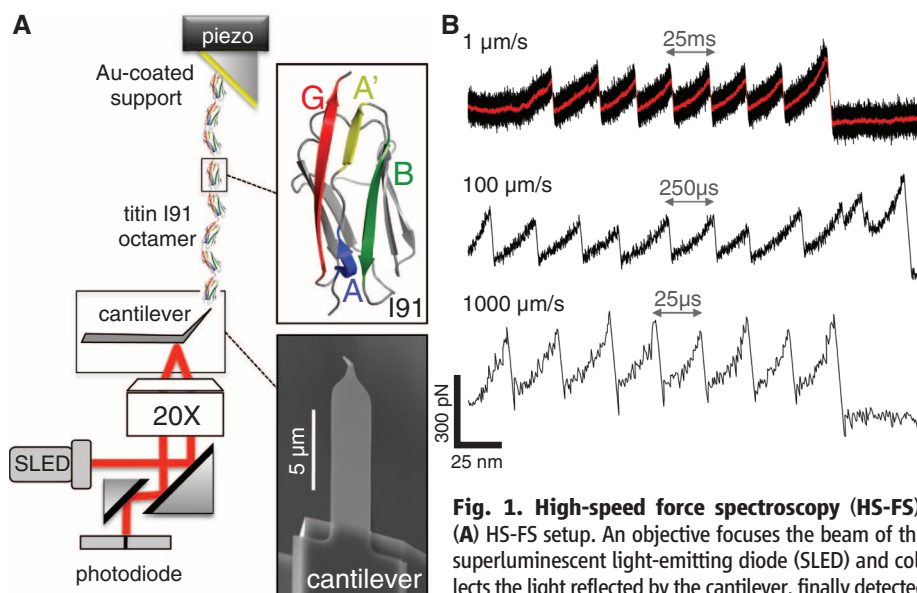


Fig. 1. High-speed force spectroscopy (HS-FS). (A) HS-FS setup. An objective focuses the beam of the superluminescent light-emitting diode (SLED) and collects the light reflected by the cantilever, finally detected by a segmented photodiode. Titin I91 concatemers of

eight domains are immobilized on a tilted gold-coated surface via C-terminal cysteines. They are pulled by their N-terminal histidine tag with a nickel-coated tip at the end of a short cantilever. Tilting the sample surface further reduces hydrodynamic forces. Top inset shows a titin I91 domain (PDB ID: 1TIT) with relevant β strands colored in blue (A), yellow (A'), green (B), and red (G). Bottom inset shows a scanning electron micrograph of a short cantilever. (B) Force-extension curves acquired at three different retraction velocities: 1, 100, and 1000 $\mu\text{m/s}$. The 1 $\mu\text{m/s}$ curve is moving average-filtered (red trace, 65- μs time window). Times to unfold a single I91 domain are indicated by arrows.

¹U1006 INSERM, Aix-Marseille Université, Parc Scientifique et Technologique de Luminy, 163 avenue de Luminy, 13009 Marseille, France. ²Bioelectronics Group, Department of Electronics, Universitat de Barcelona, c/ Martí Franques 1, 08028 Barcelona, Spain.

*Corresponding author. E-mail: simon.scheuring@inserm.fr

therefore, the slope upturn at high velocities corresponds to a shift of the barrier closer to the native state. From our data, the regime transition occurs at experimental velocities of $\sim 1000 \mu\text{m/s}$ and a critical force of $\sim 350 \text{ pN}$ (supplementary materials). Our fastest experiment at $3870 \mu\text{m/s}$ is situated at the beginning of the deterministic regime, whereas most of the HS-FS data points characterize the transition from the stochastic regime to the deterministic regime (Fig. 2B). SMD simulations at much higher velocities ($\gg 1000 \mu\text{m/s}$) have generally been carried out in this deterministic regime. Although SMD simulation-derived forces are in agreement with our fastest pulling data, the theoretically predicted trend deviates from simulations at velocities of $>10^4 \mu\text{m/s}$. These deviations may be

explained by slight differences in the simulated conditions (e.g., temperature) or by the simple cusp shape of the potential in the theory. More refined theories may be necessary to describe the unfolding at very high velocities. The model fit results in an energy landscape where the unfolding transition barrier (x_B) is located at 0.89 nm and the molecular elasticity (k_m) is 376 pN/nm , leading to an unfolding barrier height (ΔG) of $36k_B T$ and a spontaneous unfolding rate k_0 of $2 \times 10^{-10} \text{ s}^{-1}$ (fig. S4). Similar values were obtained by fitting a unified model valid for different potential shapes (28) to the unfolding forces at velocities of $\leq 100 \mu\text{m/s}$; this suggested that the reported parameters are independent of the potential shape (fig. S6). Our barrier position (0.89 nm) is larger than previous experi-

mental estimates [0.25 nm (23); 0.30 nm (5)] but is in better agreement with the distance (1.1 to 1.4 nm) at which the secondary structure of I91 breaks in simulations (8, 15). The relatively narrow range of experimental velocities in former FS experiments did not show an upturn in the force spectra and hence justified the Bell-Evans assumption of a fixed distance to the transition barrier under force. Our experiments at higher velocities show that this assumption is not valid. Furthermore, the data allowed us to estimate a diffusion coefficient of the protein along the reaction coordinate of the free energy landscape $D \approx 4 \times 10^3 \text{ nm}^2/\text{s}$ (supplementary materials). This is orders of magnitude slower than diffusion coefficients of proteins in solution ($\sim 10^8 \text{ nm}^2/\text{s}$) (29).

Fig. 2. High-speed dynamic force spectrum of titin I91. (A) Unfolding force histograms of the 1, 100, and $1000 \mu\text{m/s}$ retraction velocity experiments. (B) Average unfolding forces versus retraction velocity obtained using HS-FS, conventional FS (error bars denote SD), and steered molecular dynamics simulations [data from Lee *et al.* (15)]. Solid red line is the fit to the entire dynamic range of HS-FS with the full microscopic model (26) with fitting parameters ($\pm \text{SD}$) of $x_B = 0.89 \pm 0.05 \text{ nm}$, $D = 3925 \pm 183 \text{ nm}^2/\text{s}$, and $k_m = 376 \pm 28 \text{ pN/nm}$.

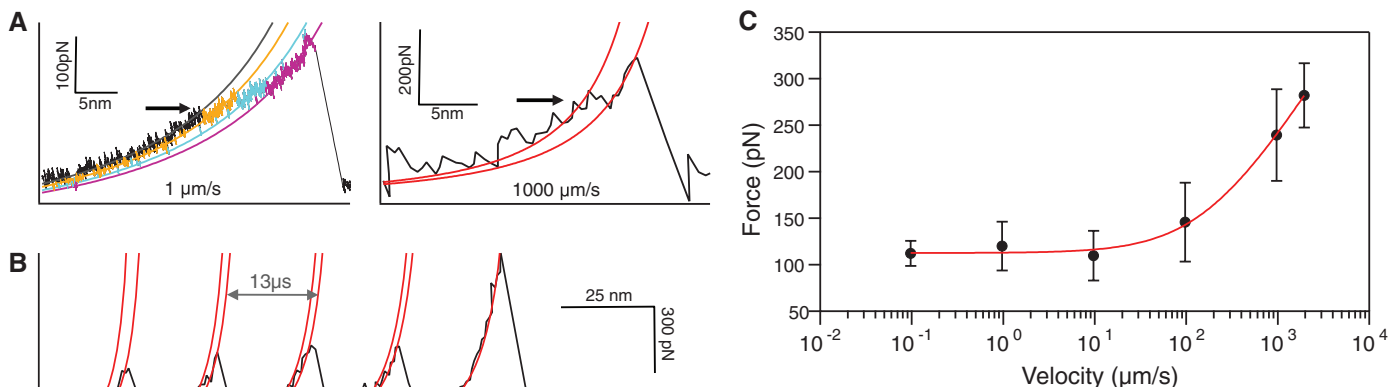
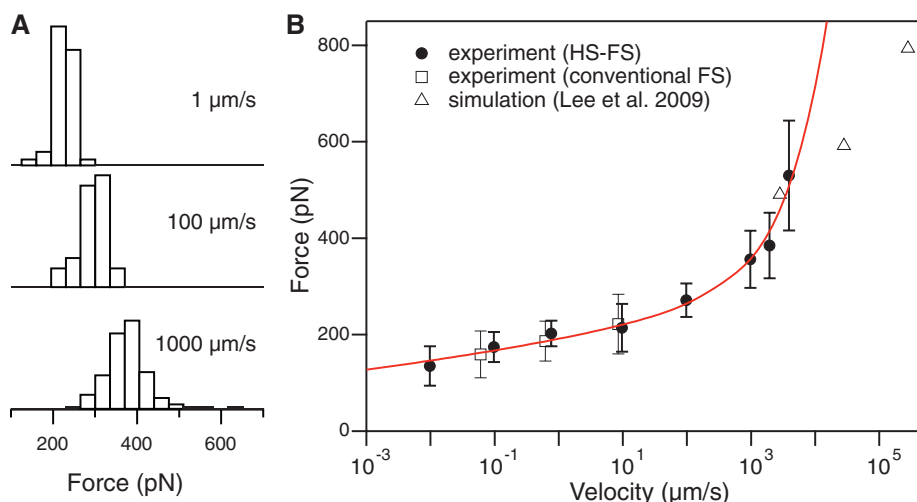


Fig. 3. High-speed force spectroscopy of unfolding intermediate. (A) Left: Force-extension trace at $1 \mu\text{m/s}$ (moving average-filtered with $65\text{-}\mu\text{s}$ time window) showing the intermediate unfolding state "hump" (arrow) separating antiparallel β strands A and B. Cantilever fluctuations above the noise level of the trace are interpreted as hopping between intermediate states of the remaining folded domains (see fig. S5). Colored lines are worm-like chain (WLC) model fits. Right: Force-extension trace (black line) at $1000 \mu\text{m/s}$ showing the intermediate unfolding state "hump" (arrow) and complete unfolding peaks. The difference between the contour lengths is consistent with a separation of $n \times 0.7 \text{ nm}$ of each A-B β -strand pair of the remaining folded domains (11). (B) Force-extension curve at $2000 \mu\text{m/s}$ showing unfolding of four I91 domains. Red lines are WLC fits to the "hump" and complete unfolding peaks. The contour length distance difference between the "hump" and the complete unfolding decreases with the decreasing number of remaining folded domains. (C) Dynamic force spectrum of the intermediate unfolding state. Solid red line is the best fit of the model developed by Friddle *et al.* (33) to the experimental data with fitting parameters ($\pm \text{SD}$) $x_t = 0.060 \pm 0.004 \text{ nm}$, $f_{eq} = 113 \pm 1 \text{ pN}$, and $k_{NI} = 6959^{+1398}_{-990} \text{ s}^{-1}$.

remaining folded domains (see fig. S5). Colored lines are worm-like chain (WLC) model fits. Right: Force-extension trace (black line) at $1000 \mu\text{m/s}$ showing the intermediate unfolding state "hump" (arrow) and complete unfolding peaks. The difference between the contour lengths is consistent with a separation of $n \times 0.7 \text{ nm}$ of each A-B β -strand pair of the remaining folded domains (11). (B) Force-extension curve at $2000 \mu\text{m/s}$ showing unfolding of four I91 domains. Red lines are WLC fits to the "hump" and complete unfolding peaks. The contour length distance difference between the "hump" and the complete unfolding decreases with the decreasing number of remaining folded domains. (C) Dynamic force spectrum of the intermediate unfolding state. Solid red line is the best fit of the model developed by Friddle *et al.* (33) to the experimental data with fitting parameters ($\pm \text{SD}$) $x_t = 0.060 \pm 0.004 \text{ nm}$, $f_{eq} = 113 \pm 1 \text{ pN}$, and $k_{NI} = 6959^{+1398}_{-990} \text{ s}^{-1}$.

Slow diffusion has been interpreted as a result of cantilever viscous damping (30) or by local minima along the unfolding pathway (31). Given the much smaller damping coefficient of the cantilevers used here, our data support the hypothesis that roughness in the free energy landscape slows unfolding. Although our estimated barrier height ($36.4k_B T$) is similar to that measured from chemical unfolding ($37k_B T$), the intrinsic unfolding rate ($2 \times 10^{-10} \text{ s}^{-1}$) is much slower than estimates from FS at slow pulling velocity ($3.3 \times 10^{-4} \text{ s}^{-1}$) and chemical unfolding ($4.9 \times 10^{-4} \text{ s}^{-1}$) (23). The fast intrinsic dissociation rate from slow FS and Bell-Evans analysis suggests an oversimplified view of forced unfolding, whereas chemical unfolding explores unrestricted unfolding pathways different from the physiologically relevant directional unfolding during muscle relaxation. Indeed, our slow k_0 suggests that the titin I91 domains unfold only very rarely at the estimated physiological forces ($\sim 5 \text{ pN}$) acting on distal titin Ig domains (32).

The use of short cantilevers with fast response (response time $\tau_c \approx 0.7 \mu\text{s}$; Fig. 1) allowed us not only to pull fast but also at conventional velocities (10 to 1000 nm/s) with microsecond time resolution. This response time is almost three orders of magnitude shorter than that of conventional cantilevers and allowed estimation of a lower limit for the relaxation time of the unfolded polypeptide chain ($< 2 \mu\text{s}$; fig. S3). Before complete domain unfolding, an intermediate state has previously been documented by the so-called “hump” in force curves (Fig. 3A, arrows). This intermediate state is characterized by a force drop in the stretching regime (Fig. 3A, arrows), caused by the separation of the A-B β -strand pair as revealed by simulations (11, 12). HS-FS measurements show separation of the A-B β -strand pair in several domains within $1 \mu\text{s}$ (Fig. 3B, first peak). Additionally, at high retraction speeds ($> 1 \text{ mm/s}$), the first domain as well as consecutive domains presented a “hump” before unfolding. The percentage of domains displaying intermediate unfolding decreased from $\sim 95\%$ at the lowest velocities to $\sim 40\%$ at the highest velocities. At 2 mm/s , the time between domain unfolding was $\sim 10 \mu\text{s}$ (Fig. 3B). Thus, this short time lapse after the unfolding of the preceding domain is sufficient for domains to refold into their native state. This result allows us to set the lower limit for the refolding rate from the intermediate to the native state to at least $\sim 10^5 \text{ s}^{-1}$, much faster than previous estimates (25 s^{-1}) (11).

We analyzed the intermediate unfolding state up to $2000 \mu\text{m/s}$; beyond this velocity, it is difficult to assess an accurate measurement (Fig. 3 and fig. S3). At conventional pulling velocities, the average unfolding forces to the intermediate state are independent of pulling rate. At velocities faster than $\sim 100 \mu\text{m/s}$, average “hump” forces increase drastically, reaching values up to $\sim 300 \text{ pN}$ (Fig. 3C), consistent with “hump” forces observed in simulations (15). The slow pulling regime ($< 100 \mu\text{m/s}$) is dominated by near-equilibrium

unfolding and refolding of the A-B β -strand pair and defines the equilibrium force (Fig. 3A). At higher velocities, refolding of the A-B β -strand pair is negligible and the structure unfolds stochastically at forces that increase with the logarithm of the pulling rate (33) (Fig. 3C and supplementary materials). The model fitted our results with an unfolding rate at zero force from native to intermediate (k_{NI}^0) of $7 \times 10^3 \text{ s}^{-1}$, an even faster folding rate at zero force from intermediate to native (k_{IN}^0) of $4 \times 10^5 \text{ s}^{-1}$, a distance x_t to the transition barrier of only 0.06 nm , and an equilibrium force f_{eq} of 113 pN where $k_{\text{NI}}^0 = k_{\text{IN}}^0 = 2.8 \times 10^4 \text{ s}^{-1}$. This results in an equilibrium free energy difference between the native and intermediate states of $\sim 4.1k_B T$, in agreement with the expected energy of three hydrogen bonds.

Although the absolute values of the calculated rates should be interpreted with care, the refolding rate of $4 \times 10^5 \text{ s}^{-1}$ is in excellent agreement with the lower limit ($\sim 10^5 \text{ s}^{-1}$) determined directly through observation of reformed A-B β -strand pairs in high-velocity unfolding traces (fig. S3). This suggests fast dynamic equilibrium of unfolding and refolding of β strand pair A and B at pulling forces up to $\sim 100 \text{ pN}$, probably maintained by the antiparallel structure. This behavior has been observed in SMD runs (12, 15). Furthermore, a recent computational small protein folding study reported that an antiparallel three- β -strand domain required an average of $21 \mu\text{s}$ to fold (34). This suggests a novel insight into the A-B β -strand pair and perhaps into short β folds in general: unfolding at fast rates, and refolding at even faster rates, as a feature of their structural equilibrium.

The combination of SMD with experimental FS has been important in understanding protein unfolding and mechanical stability. Our HS-FS methodology provides pulling velocities over six orders of magnitude and provides microsecond time resolution, achieving rates comparable to SMD simulations and thus allowing direct comparison of experimental and simulated unfolding forces. We expect that the now accessible dynamic range of HS-FS will stimulate the development of novel theories. Our results imply detailed mechanisms of the various steps during titin I91 unfolding: At zero and moderate forces, the protein fluctuates between the native and intermediate states. Under increasing force, only the intermediate state is populated. Thus, the tethered molecule reveals slow diffusion along the unfolding pathway, which, in combination with a high energy barrier, results in high mechanical stability. Direct comparison of FS and SMD simulations will likely provide new insights into other important biological processes such as lipid membrane dynamics (35) and receptor-ligand unbinding (7, 9).

References and Notes

1. D. S. Herman *et al.*, *N. Engl. J. Med.* **366**, 619–628 (2012).
2. K. Maruyama, S. Matsubara, R. Natori, Y. Nonomura, S. Kimura, *J. Biochem.* **82**, 317–337 (1977).

3. M. S. Z. Kellermyer, S. B. Smith, H. L. Granzier, K. Bustamante, *Science* **276**, 1112–1116 (1997).
4. L. Tskhovrebova, J. Trinick, J. A. Sleep, R. M. Simmons, *Nature* **387**, 308–312 (1997).
5. M. Rief, M. Gautel, F. Oesterhelt, J. M. Fernandez, H. E. Gaub, *Science* **276**, 1109–1112 (1997).
6. H. Grubmüller, B. Heymann, P. Tavan, *Science* **271**, 997 (1996).
7. S. Izrailev, S. Stepaniants, M. Balsara, Y. Oono, K. Schulten, *Biophys. J.* **72**, 1568–1581 (1997).
8. H. Lu, B. Isralewitz, A. Krammer, V. Vogel, K. Schulten, *Biophys. J.* **75**, 662–671 (1998).
9. E. L. Florin, V. T. Moy, H. E. Gaub, *Science* **264**, 415–417 (1994).
10. G. U. Lee, L. A. Chrisey, R. J. Colton, *Science* **266**, 771–773 (1994).
11. P. E. Marszalek *et al.*, *Nature* **402**, 100–103 (1999).
12. H. Lu, K. Schulten, *Biophys. J.* **79**, 51–65 (2000).
13. S. B. Fowler *et al.*, *J. Mol. Biol.* **322**, 841–849 (2002).
14. R. B. Best *et al.*, *J. Mol. Biol.* **330**, 867–877 (2003).
15. E. H. Lee, J. Hsin, M. Sotomayor, G. Comellas, K. Schulten, *Structure* **17**, 1295–1306 (2009).
16. T. Ando *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12468–12472 (2001).
17. T. Uchihashi, R. Iino, T. Ando, H. Noji, *Science* **333**, 755–758 (2011).
18. N. Koder, D. Yamamoto, R. Ishikawa, T. Ando, *Nature* **468**, 72–76 (2010).
19. I. Casuso *et al.*, *Nat. Nanotechnol.* **7**, 525–529 (2012).
20. T. Ando, T. Uchihashi, T. Fukuma, *Prog. Surf. Sci.* **83**, 337–437 (2008).
21. M. B. Viani *et al.*, *J. Appl. Phys.* **86**, 2258 (1999).
22. R. B. Best *et al.*, *Anal. Chim. Acta* **479**, 87–105 (2003).
23. M. Carrion-Vazquez *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3694–3699 (1999).
24. R. Merkel, P. Nassoy, A. Leung, K. Ritchie, E. Evans, *Nature* **397**, 50–53 (1999).
25. X. H. Zhang, E. Wojcikiewicz, V. T. Moy, *Biophys. J.* **83**, 2270–2279 (2002).
26. G. Hummer, A. Szabo, *Biophys. J.* **85**, 5–15 (2003).
27. Z. T. Yew, M. Schlierf, M. Rief, E. Paci, *Phys. Rev. E* **81**, 031923 (2010).
28. O. K. Dudko, G. Hummer, A. Szabo, *Phys. Rev. Lett.* **96**, 108101 (2006).
29. L. J. Lapidus, W. A. Eaton, J. Hofrichter, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7220–7225 (2000).
30. R. Berkovich *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 14416–14421 (2012).
31. H. Lannon, J. S. Haghighpanah, J. K. Montclare, E. Vanden-Eijnden, J. Bruijic, *Phys. Rev. Lett.* **110**, 128301 (2013).
32. H. Li *et al.*, *Nature* **418**, 998–1002 (2002).
33. R. W. Friddle, A. Noy, J. J. De Yoreo, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 13573–13578 (2012).
34. K. Lindorff-Larsen, S. Piana, R. O. Dror, D. E. Shaw, *Science* **334**, 517–520 (2011).
35. L. Redondo-Morata, M. I. Giannotti, F. Sanz, *Langmuir* **28**, 6403–6410 (2012).

Acknowledgments: We thank A. Sastre and J. Otero for helpful discussions. Supported by European Research Council starting grant 310080 and Agence Nationale de la Recherche grant ANR-12-BS10-009-01. L.G. was a recipient of travel grants (FPI grant BES-2010-031186 from the Spanish Ministerio de Economía y Competitividad and STSM COST Action TD1002-10006).

Supplementary Materials

www.sciencemag.org/content/342/6159/741/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S6
References (36–39)

29 April 2013; accepted 30 September 2013
10.1126/science.1239764

Coordinated Effects of Sequence Variation on DNA Binding, Chromatin Structure, and Transcription

Helena Kilpinen,^{1,2,3*} Sebastian M. Waszak,^{2,4*} Andreas R. Gschwind,^{2,5*} Sunil K. Raghav,⁴ Robert M. Witwicki,⁵ Andrea Orioli,⁵ Eugenia Migliavacca,^{2,5} Michaël Wiedeker,⁵ Maria Gutierrez-Arcelus,^{1,2,3} Nikolaos I. Panousis,^{1,2,3} Alisa Yurovsky,^{1,2,3} Tuuli Lappalainen,^{1,2,3} Luciana Romano-Palumbo,¹ Alexandra Planchon,¹ Deborah Bielser,¹ Julien Bryois,^{1,2,3} Ismael Padioleau,^{1,2,3} Gilles Udin,⁴ Sarah Thurnheer,⁶ David Hacker,⁶ Leighton J. Core,⁷ John T. Lis,⁷ Nouria Hernandez,^{5†} Alexandre Reymond,^{5†} Bart Deplancke,^{2,4†} Emmanouil T. Dermitzakis^{1,2,3,8†}

DNA sequence variation has been associated with quantitative changes in molecular phenotypes such as gene expression, but its impact on chromatin states is poorly characterized. To understand the interplay between chromatin and genetic control of gene regulation, we quantified allelic variability in transcription factor binding, histone modifications, and gene expression within humans. We found abundant allelic specificity in chromatin and extensive local, short-range, and long-range allelic coordination among the studied molecular phenotypes. We observed genetic influence on most of these phenotypes, with histone modifications exhibiting strong context-dependent behavior. Our results implicate transcription factors as primary mediators of sequence-specific regulation of gene expression programs, with histone modifications frequently reflecting the primary regulatory event.

Functional genomic elements have been linked to specific chromatin signatures in different cell types (1), illustrating control of transcriptional processes through multiple layers of genome organization. Although allele-specific gene expression is widespread (2), it has been difficult to pinpoint the upstream cis-regulatory variants and how they affect chromatin states. We performed chromatin immunoprecipitation (ChIP) of five histone posttranslational modifications (hPTMs) (H3K4me1, H3K4me3, H3K27ac, H3K27me3, and H4K20me1), three transcription factors (TFs) (TFIIB, PU.1, and MYC), and the second largest RNA polymerase II subunit RPB2 (POLR2B) (fig. S1) in lymphoblastoid cell lines (LCLs) derived from two parent-offspring trios (3). A subset of the ChIP assays was also performed in eight additional unrelated individuals. We further profiled one of the trios with global run-on sequencing (GRO-seq), which measures nascent transcription at all transcribed regions (fig. S2), and examined available deoxyribonuclease I (DNase I)-seq and

CCCTC-binding factor (CTCF) ChIP-seq data (4). All 14 individuals were additionally profiled for mRNA expression (5). Clustering of the molecular phenotypes along promoters and enhancers was consistent with published reports (1) (figs. S3 to S5).

We identified sites of allele-specific (AS) TF binding, hPTM, and transcription for all assays (5), ranging from 11 to 12% for TFs (4, 6) to 6 to 30% for hPTMs at heterozygous sites accessible for the analysis (median across all individuals) (Fig. 1A and fig. S6). Notably, in the two trios, fewer AS effects were observed in mRNA (mRNA-seq, 5%) than in nascent transcripts (GRO-seq, 27 to 28%) (5), likely reflecting posttranscriptional modifications.

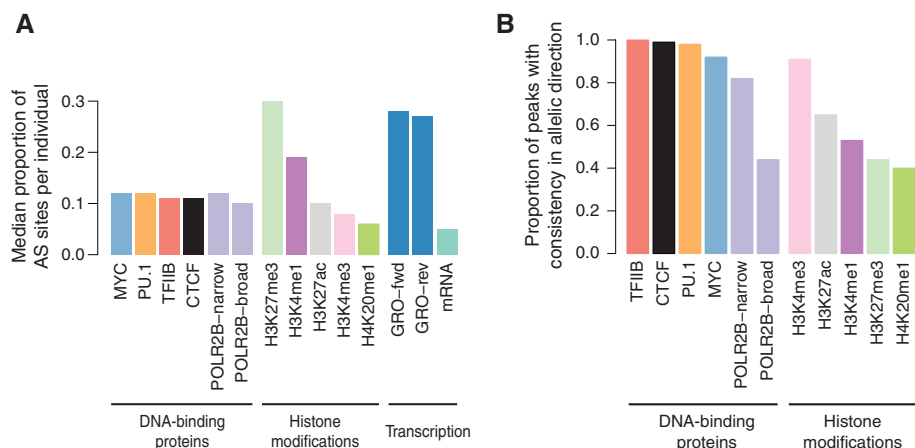


Fig. 1. Allele-specific (AS) activity within transcriptional and chromatin layers. (A) Proportion of accessible heterozygous SNP sites showing significant AS activity (median across all individuals, $n = 3$ to 14). **(B)** Consistency of allelic effects within genomic regions of TF binding and histone modification. Bars represent the proportion of peaks with a consistent allelic direction at two or more SNP sites.

Multiple heterozygous single-nucleotide polymorphisms (SNPs) overlapping regions of TF activity showed high consistency in allelic direction within individuals (Fig. 1B and fig. S7, A and B). Allelic consistency in nascent transcripts and histone modifications was high even with sites several kilobases apart and decreased with genomic distance (logistic regression, $P < 0.05$; fig. S7C). The strongest AS effects were enriched at promoters, whereas the allelic signals of marks of enhancer activity (PU.1, H3K4me1, H3K27ac) or heterochromatin (H3K27me3) showed a more dispersed distribution (fig. S8). We also analyzed all accessible heterozygous SNPs overlapping known expression quantitative trait loci (eQTLs) from the 1000 Genomes phase 1 populations (5, 7) and observed an enrichment of allelic bias at eQTLs relative to non-eQTLs for TFs ($P = 0.016$, Mann-Whitney U test) but not for hPTMs (fig. S9); this finding suggests that a TF binding change is often causal to the gene expression change.

Linking hPTM signatures with specific DNA sequence features has proven difficult (8), but for sequence-specific TFs it is possible to assess whether the observed AS effects are due to motif-disrupting variants (fig. S10). Categorization of significant AS binding sites, with respect to predicted TF motifs, revealed three classes of binding SNPs (B-SNPs): B-SNPs located either within (class I) or adjacent to (class II) predicted PU.1 and MYC consensus TF motifs, or B-SNPs in motif-devoid peaks (class III). Class I sites were enriched for B-SNPs relative to the other two classes (fig. S11, A and B, for PU.1; fig. S12, A and B, for MYC), which suggests that SNP-mediated disruption of the TF motif is likely causal to the observed AS binding activity. However, most TF AS binding events (70%, PU.1; 97%, MYC) appear triggered through TF consensus motif-independent mechanisms (figs. S11A and S12A) (6, 9). For example, allelic binding cooperativity tests (5) revealed four additional motifs

¹Department of Genetic Medicine and Development, University of Geneva Medical School, 1211 Geneva, Switzerland.

²Swiss Institute of Bioinformatics, 1015 Lausanne, Switzerland.

³Institute of Genetics and Genomics of Geneva, University of Geneva, 1211 Geneva, Switzerland. ⁴Laboratory of Systems Biology and Genetics, Institute of Bioengineering, School of Life Sciences, Swiss Federal Institute of Technology (EPFL), 1015 Lausanne, Switzerland. ⁵Center for Integrative Genomics, Faculty of Biology and Medicine, University of Lausanne, 1015 Lausanne, Switzerland. ⁶Protein Expression Core Facility, School of Life Sciences, Swiss Federal Institute of Technology (EPFL), 1015 Lausanne, Switzerland. ⁷Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14850, USA. ⁸Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah 21589, Saudi Arabia.

*These authors contributed equally to this work.
†Corresponding author. E-mail: emmanouil.dermitzakis@unige.ch (E.T.D.); bart.deplancke@epfl.ch (B.D.); alexandre.reymond@unil.ch (A.R.); nouria.hernandez@unil.ch (N.H.)

(NFKB1, POU2F2, PRDM1, and STAT2), located proximal to the PU.1-bound site, that show covariance with AS PU.1 binding activity [false discovery rate (FDR) = 5%; Fig. 2A and fig. S13] and collectively explain another 7.5% of AS PU.1 binding activity.

Despite a strong correlation between motif score differences and AS binding (figs. S11C and S12C; >90% expected direction), we observed that the majority of motif-disrupting SNPs do not show significant allelic effects (figs. S11A and S12A). Therefore, we tested whether homotypic

TF motifs (i.e., multiple motifs for the same TF) located within PU.1-bound regions might buffer the effects of motif-disrupting SNPs (5, 10, 11) and found that TF-bound regions with homotypic motifs exhibit fewer allelic effects (41% versus 25%; $P = 0.0087$, Mann-Whitney U test). In addition, the impact of SNPs on TF motifs scales with the likelihood of observing significant AS effects (Fig. 2B and fig. S12D), but this trend is not significant if a second, unaffected homotypic TF motif is located nearby (Fig. 2B and fig. S11D). These results suggest that homotypic motif clusters buffer the effect of genetic variation over several similar binding sites.

We investigated the genetic component of allele-specific chromatin and binding signals by (i) comparing the direction of allelic bias at shared significant AS sites across 10 unrelated individuals (Fig. 3A), and (ii) testing for transmission of allelic effects from parents to children (Fig. 3B and fig. S17) (4). Allelic directions at shared significant AS sites in the unrelated individuals were significantly correlated ($P < 0.05$, Spearman correlation; fig. S16A), with mRNA showing the highest degree of consistency in allelic directions between individuals, followed by TF binding and histone modification, respectively (Fig. 3A and figs. S14 to S16). We observed evidence of

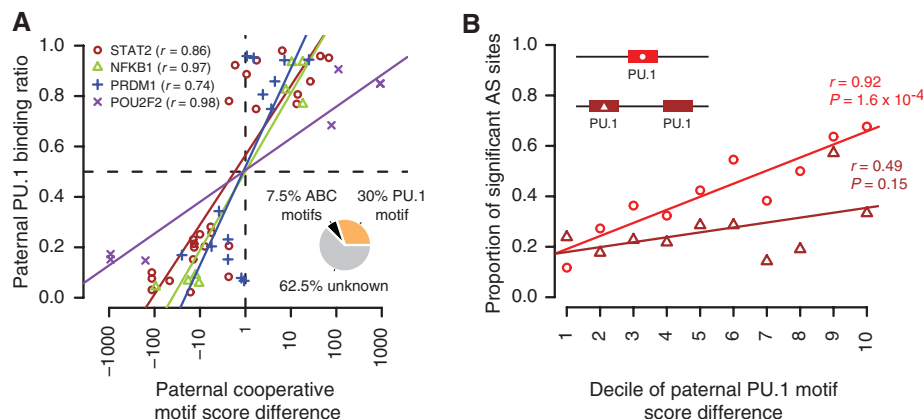


Fig. 2. DNA sequence properties at allele-specific PU.1 binding sites. (A) SNPs in PU.1-bound and cooperative TF motifs are predictive of AS PU.1 binding (5% FDR) (5). (B) PU.1-bound regions (peaks) with homotypic PU.1 motifs show a weak response toward motif-disrupting SNPs. Motif-disrupting SNPs were split into two classes (one or two PU.1 motifs per peak) and grouped according to their motif impact (1, lowest; 10 highest).

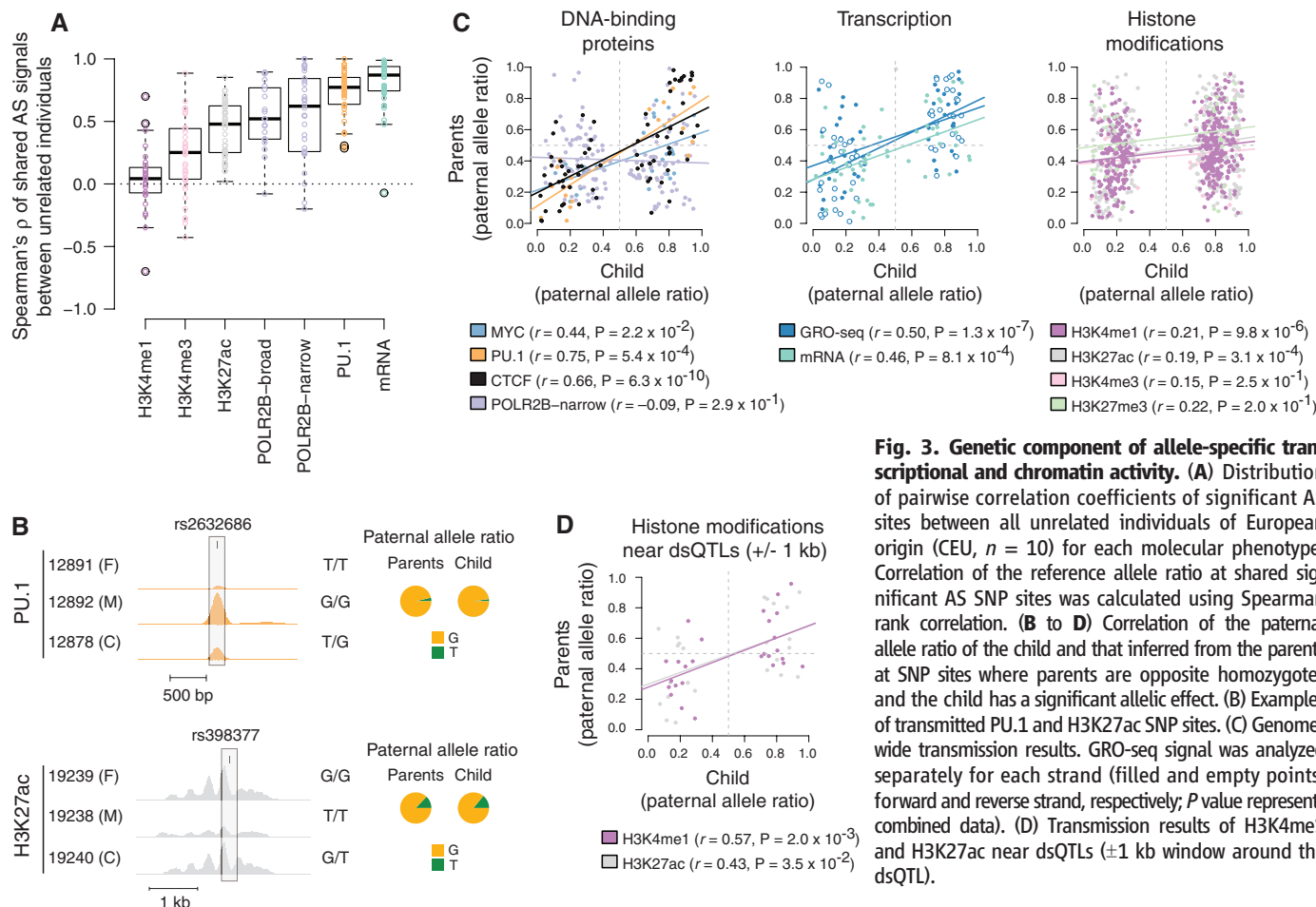


Fig. 3. Genetic component of allele-specific transcriptional and chromatin activity. (A) Distribution of pairwise correlation coefficients of significant AS sites between all unrelated individuals of European origin (CEU, $n = 10$) for each molecular phenotype. Correlation of the reference allele ratio at shared significant AS SNP sites was calculated using Spearman rank correlation. (B to D) Correlation of the paternal allele ratio of the child and that inferred from the parents at SNP sites where parents are opposite homozygotes and the child has a significant allelic effect. (B) Examples of transmitted PU.1 and H3K27ac SNP sites. (C) Genome-wide transmission results. GRO-seq signal was analyzed separately for each strand (filled and empty points, forward and reverse strand, respectively; P value represents combined data). (D) Transmission results of H3K4me1 and H3K27ac near dsQTLs (± 1 kb window around the dsQTL).

significant parental transmission with all three regulatory TFs ($\rho = 0.44$ to 0.75 , $P \leq 0.02$, Spearman correlation; Fig. 3C and fig. S17), consistent with their strong sequence dependence (4, 6). For hPTMs, evidence of transmission was detected for the active histone marks H3K4me1, H3K4me3, and H3K27ac ($\rho = 0.12$ to 0.21 ; $P \leq 0.02$), but their level of transmission was lower than for TFs. Transmission signals for mRNA levels and nascent transcription were significant and comparable to TFs ($\rho = 0.46$ and 0.50 ; $P = 0.0008$ and $P = 1.3 \times 10^{-7}$, respectively). We observed only weak transmission for POLR2B (fig. S17), possibly due to the distinct activity states of the polymerase (12). We determined the genetic control of the transmission signal of histone marks at known eQTLs (7) and DNase I sensitivity QTLs (dsQTLs) (13), respectively, because the former are enriched within TF binding sites (13). Transmission of the active marks H3K4me1, H3K4me3, and H3K27ac was stronger near eQTLs and dsQTLs ($\rho = 0.31$ to 0.57) than genome-wide (Fig. 3D and fig. S20), which suggests that the transmission behavior of the overall chromatin state depends on the properties of the underlying sequence. Collectively, these findings indicate coordinated and genetically driven changes between TF binding and histone modifications, and suggest that TFs are the primary determinants of regulatory interactions (14–16).

To further assess the extent of allelic coordination (AC) between distinct genomic regulatory layers, we calculated the correlation between AS effects across pairs of molecular phenotypes (fig. S21). We observed that each testable phenotype

exhibits significant correlation in allelic ratios with one or multiple phenotypes (Spearman correlation, $P < 0.05$). The majority of AC events reflect relationships between distinct regulatory layers that have also been observed quantitatively [e.g., POLR2B–H3K4me3 at promoters (1, 17, 18); GRO-seq–H3K4me1–H3K27ac at putative enhancers (19)] (Fig. 4A and fig. S21). These results support a strong allelic (i.e., local) interconnectivity among regulatory and general TFs, histone modifications, and transcription.

eQTLs are often located distal to their target genes (20), indicating that allelic signals within regulatory layers might extend over short and long distances. We examined haplotypic coordination (HC)—defined as long-range coordination in allelic direction on the same chromosome—of AS effects at nonoverlapping heterozygous sites (5) (Fig. 4B and fig. S21), and found that every TF and histone mark exhibits HC with one or more regulatory layer(s) around genes and their flanking regions (fig. S21; Spearman correlation, $P < 0.05$). The degree of coordination varied between regulatory layers, ranging from -0.24 (GRO-seq–CTCF; $P = 0.03$) to 0.64 (MYC–mRNA; $P = 2.9 \times 10^{-8}$). The majority (>90%) of significant HC events were positive; that is, the allelic bias co-occurred on the same haplotype (Fig. 4B and fig. S21). For 25% of assay pairs tested, the strength of HC was significantly correlated with the genomic distance between SNP pairs (logistic regression, $P < 0.05$; odds ratio = 0.19 to 2.2) (fig. S22). For example, the enhancer-associated histone marks H3K4me1 and H3K27ac showed allelic consistency up to 200 kb with the TF PU.1. Thus,

a single or few variant(s) likely trigger long-distance allelic effects over many of the regulatory layers acting on a genomic region.

Our work has revealed abundant allele-specific activity across all regulatory layers. Parental transmission of the allelic effects suggests that DNA sequence variations affecting transcription, TF binding, and histone modifications are largely transmitted from parents to children, with allelic histone effects showing more context-dependent behavior compared to TFs. Coordinated allelic and haplotypic behavior at different functional elements of the genome suggest that TF binding, histone modifications, and transcription operate within the same allelic framework. This is consistent with the fact that a few TFs can induce cellular reprogramming and massive changes in the chromatin landscape (21), and that the maintenance of a transcription-permissive environment and transcriptional memory are independent of histone modifications (22). Both histone modifications and TF binding are under genetic control, but histone modifications are more prone to stochastic, possibly transient effects and likely reflect (23), rather than define, coordinated regulatory interactions.

References and Notes

1. B. E. Bernstein *et al.*, *Nature* **489**, 57–74 (2012).
2. S. B. Montgomery *et al.*, *Nature* **464**, 773–777 (2010).
3. G. R. Abecasis *et al.*, *Nature* **467**, 1061–1073 (2010).
4. R. McDaniel *et al.*, *Science* **328**, 235–239 (2010).
5. See supplementary materials on Science Online.
6. T. E. Reddy *et al.*, *Genome Res.* **22**, 860–869 (2012).
7. T. Lappalainen *et al.*, *Nature* **501**, 506–511 (2013).
8. S. Henikoff, A. Shilatifard, *Trends Genet.* **27**, 389–396 (2011).
9. M. Kasowski *et al.*, *Science* **328**, 232–235 (2010).
10. M. T. Maurano, H. Wang, T. Kutayin, J. A. Stamatoyannopoulos, *PLoS Genet.* **8**, e1002599 (2012).
11. M. Spivakov *et al.*, *Genome Biol.* **13**, R49 (2012).
12. L. J. Core, J. J. Waterfall, J. T. Lis, *Science* **322**, 1845–1848 (2008).
13. J. F. Degner *et al.*, *Nature* **482**, 390–394 (2012).
14. D. H. Erwin, E. H. Davidson, *Nat. Rev. Genet.* **10**, 141–148 (2009).
15. O. Hobert, *Curr. Biol.* **21**, R146–R147 (2011).
16. M. Ptashne, *Curr. Biol.* **17**, R233–R236 (2007).
17. C. Y. Lin *et al.*, *Cell* **151**, 56–67 (2012).
18. Z. Nie *et al.*, *Cell* **151**, 68–79 (2012).
19. D. Wang *et al.*, *Nature* **474**, 390–394 (2011).
20. E. Grundberg *et al.*, *Nat. Genet.* **44**, 1084–1089 (2012).
21. C. A. Doege *et al.*, *Nature* **488**, 652–655 (2012).
22. R. Margueron, D. Reinberg, *Nat. Rev. Genet.* **11**, 285–296 (2010).
23. M. Gutierrez-Arcelus *et al.*, *Elife* **2**, e00523 (2013).

Acknowledgments: Supported by Swiss National Science Foundation grants CRSI33_130326 (E.T.D., B.D., A.R., N.H.), 31003A_132958 (N.H.), 31003A_130342 (E.T.D.), and 31003A_129835 (A.R.); the European Research Council (E.T.D.); the Louis-Jeantet Foundation (E.T.D.); European Molecular Biology Organization fellowship ALTf 2010-337 (H.K.); a fellowship from the doctoral school of the Faculty of Biology and Medicine, University of Lausanne (R.M.W.); the NCCR Frontiers in Genetics Program (M.G.-A., J.B., E.T.D., B.D.); the Japanese-Swiss Science and Technology Cooperation

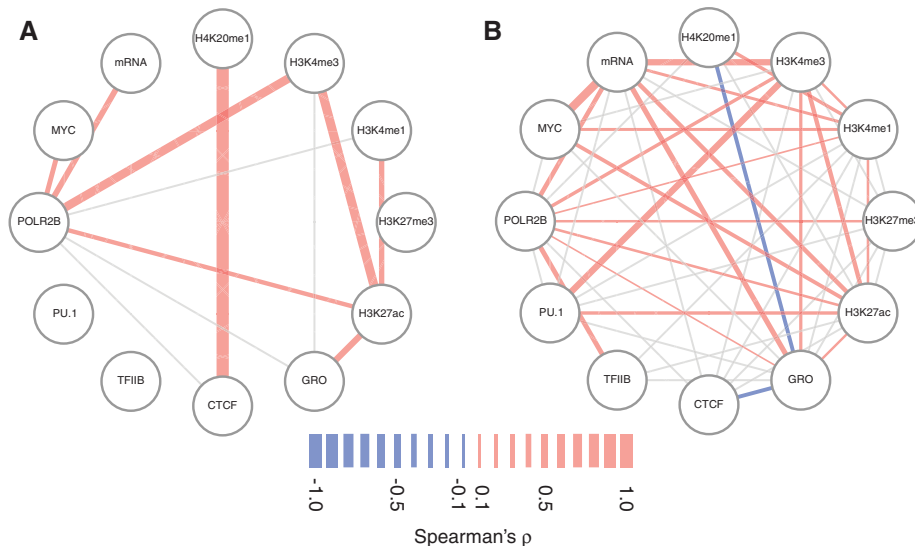


Fig. 4. Local, short- and long-range coordination between transcriptional and chromatin layers. Results of allelic coordination (A) and haplotypic coordination (B) analysis at gene regions (genes ± 50 kb) (5). Coordination of the allelic effect was considered between all pairs of assays. SNP sites within genomic regions were required to show a significant AS effect in both assays. Only assay pairs with ≥ 20 SNPs were considered for the analysis. Significant Spearman rank correlation coefficients ($P < 0.05$) between the paternal allele ratios of the SNP pairs are indicated with colored lines ranging in intensity from $\rho = -1.0$ (blue) to $\rho = 1.0$ (red). Nonsignificant correlations are indicated with gray lines; missing lines indicate lack of sufficient data points for analysis.

Program (Japan Science and Technology Agency/ETH Zürich (B.D.); École Polytechnique Fédérale de Lausanne (B.D.); and NIH grants HG004845 and GM25232 (J.T.L.). The computations were performed at the Vital-IT (www.vital-it.ch) Center for High-Performance Computing of the Swiss Institute of Bioinformatics. All data in this publication are available through ArrayExpress (www.ebi.ac.uk/arrayexpress/) under accession

numbers E-MTAB-1883 (RNA-seq), E-MTAB-1884 (ChIP-seq), and E-MTAB-1885 (GRO-seq). The authors declare no competing financial interests.

Supplementary Materials

www.sciencemag.org/content/342/6159/744/suppl/DC1
Materials and Methods

Figs. S1 to S31
Tables S1 and S2
References (24–39)

26 June 2013; accepted 12 September 2013

Published online 16 October 2013;
10.1126/science.1242463

Identification of Genetic Variants That Affect Histone Modifications in Human Cells

Graham McVicker,^{1,2*} Bryce van de Geijn,^{1,3*} Jacob F. Degner,^{1,3} Carolyn E. Cain,¹ Nicholas E. Banovich,¹ Anil Raj,^{1,4} Noah Lewellen,² Marsha Myrthil,² Yoav Gilad,^{1†} Jonathan K. Pritchard^{1,2,4,5†}

Histone modifications are important markers of function and chromatin state, yet the DNA sequence elements that direct them to specific genomic locations are poorly understood. Here, we identify hundreds of quantitative trait loci, genome-wide, that affect histone modification or RNA polymerase II (Pol II) occupancy in Yoruba lymphoblastoid cell lines (LCLs). In many cases, the same variant is associated with quantitative changes in multiple histone marks and Pol II, as well as in deoxyribonuclease I sensitivity and nucleosome positioning. Transcription factor binding site polymorphisms are correlated overall with differences in local histone modification, and we identify specific transcription factors whose binding leads to histone modification in LCLs. Furthermore, variants that affect chromatin at distal regulatory sites frequently also direct changes in chromatin and gene expression at associated promoters.

Variation at noncoding regulatory sequences contributes to the genetics of complex traits (1–3), yet we still have limited understanding of the primary mechanisms by which they act. One possibility is that regulatory variants affect histone modifications that have downstream consequences on chromatin remodeling or transcription (4). There are many possible post-translational modifications of histones (i.e., “histone marks”) (4), and sets of these co-occur in distinct chromatin states (5–9), are associated with functional elements (2, 10, 11), and are sensitive indicators of changes in gene regulation (9, 12). However, we still do not know whether histone modifications are generally a cause or a consequence of gene regulation or which DNA elements direct cell type–appropriate histone marking (7, 13). Thus, studies of genetic variants that disrupt transcription factor binding sites (TFBSs) may illuminate whether histone modifications enable transcription factor binding or whether the binding of transcription factors results in histone modification.

We performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) for RNA polymerase II (Pol II) and four posttranslational modifications of histone H3 (H3K4me1, H3K4me3, H3K27ac, and H3K27me3) in 10 unrelated Yoruba lymphoblastoid cell lines (LCLs). H3K4me3 (trimethylation of lysine 4) is primarily associated with active promoters; H3K4me1 (monomethylation of lysine 4) is associated with active chromatin outside of promoters (e.g., enhancers); H3K27ac (acetylation of lysine 27) is associated with both active promoters and enhancers (6, 14); and H3K27me3 (trimethylation of lysine 27) is associated with silencing by the polycomb repressive complex 2 (PRC2) (15, 16). We mapped the ChIP-seq reads to the human genome, controlling for mapping biases introduced by polymorphic sites (17). Comparisons with ENCODE (1) showed consistent distributions of each mark (fig. S1).

To identify genetic associations with histone marks and Pol II, we developed a “combined haplotype test” that uses both read depth and allelic imbalance to enable mapping of cis-quantitative trait loci (QTLs) with small sample sizes (17). We applied the combined haplotype test to hundreds of thousands of polymorphic sites with sufficient read depth (i.e., sites within ChIP-seq peaks) and identified more than 1200 histone mark and Pol II QTLs at a false discovery rate (FDR) of 20% (Fig. 1, A and B, and fig. S3). After merging overlapping regions, we identified a total of 27 distinct QTLs for H3K4me1, 469 for

H3K4me3, 730 for H3K27ac, 118 for Pol II, and 2 for H3K27me3 (which tends not to fall into strong peaks) (table S2). At an FDR threshold of 10%, we identified 582 distinct histone mark and Pol II QTLs (table S2). In principle, some of these signals might be due to imprinting (8) or random allelic inactivation; however, several lines of evidence indicate that most of the regions that we identify are conventional QTLs (supplemental text).

Many of the histone mark QTLs overlap previously identified QTLs for deoxyribonuclease (DNase I) sensitivity (denoted “dsQTLs”) (18). DNase I sensitivity is an indicator of open chromatin, and DNase I hypersensitive sites (DHSs) typically mark active regulatory regions that are associated with active histone marks and transcription factor binding (19). Indeed, we found an enrichment of low *P* values when testing for QTL associations with Pol II and all four histone marks at dsQTLs, compared to the genome-wide set of tested single-nucleotide polymorphisms (SNPs) (Fig. 1, A and B, and fig. S3). Nevertheless, although most histone mark and Pol II QTLs are within 1 kb of a DHS (table S5), many are far from known dsQTLs (fig. S8). This suggests that histone modifications may provide more power to detect differences in chromatin state beyond that of DNase I sensitivity.

We plotted aggregate ChIP-seq read depth around DHSs associated with dsQTLs (Fig. 2), grouping read counts according to whether an individual carries the genotype associated with high, medium, or low sensitivity at a dsQTL. Most of the dsQTLs lie outside promoters, and the average histone mark read depths at dsQTL DHSs follow qualitative expectations for distal enhancers (6), with higher levels of H3K4me1 and lower levels of H3K4me3 and Pol II as compared to promoters. High-sensitivity genotypes tend to have reduced nucleosome occupancy within the DHS (20); higher levels of transcription factor binding (18); higher levels of the active marks H3K4me1, H3K4me3, and H3K27ac; and higher Pol II occupancy. The relation between DNase I and the repressive mark H3K27me3 is more complicated, as we find both positive and negative associations. We find no opposite-direction effects between DNase I and either H3K4me1, H3K4me3, or H3K27ac (fig. S5).

At expression QTLs (eQTLs) (21), we stratified the samples by the genotype of the most significant eQTL SNP and found overall patterns similar to those at dsQTLs (Fig. 2). Individuals who are homozygous for the high-expression genotype generally have higher levels of DNase I sensitivity, H3K4me3, H3K27ac, and Pol II

¹Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA. ²Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA. ³Committee on Genetics, Genomics and Systems Biology, University of Chicago, Chicago, IL 60637, USA. ⁴Department of Genetics, Stanford University, Stanford, CA 94305, USA. ⁵Department of Biology, Stanford University, Stanford, CA 94305, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: gilad@uchicago.edu (Y.G.); pritch@stanford.edu (J.K.P.)

Program (Japan Science and Technology Agency/ETH Zürich (B.D.); École Polytechnique Fédérale de Lausanne (B.D.); and NIH grants HG004845 and GM25232 (J.T.L.). The computations were performed at the Vital-IT (www.vital-it.ch) Center for High-Performance Computing of the Swiss Institute of Bioinformatics. All data in this publication are available through ArrayExpress (www.ebi.ac.uk/arrayexpress/) under accession

numbers E-MTAB-1883 (RNA-seq), E-MTAB-1884 (ChIP-seq), and E-MTAB-1885 (GRO-seq). The authors declare no competing financial interests.

Supplementary Materials

www.sciencemag.org/content/342/6159/744/suppl/DC1
Materials and Methods

Figs. S1 to S31
Tables S1 and S2
References (24–39)

26 June 2013; accepted 12 September 2013

Published online 16 October 2013;
10.1126/science.1242463

Identification of Genetic Variants That Affect Histone Modifications in Human Cells

Graham McVicker,^{1,2*} Bryce van de Geijn,^{1,3*} Jacob F. Degner,^{1,3} Carolyn E. Cain,¹ Nicholas E. Banovich,¹ Anil Raj,^{1,4} Noah Lewellen,² Marsha Myrthil,² Yoav Gilad,^{1†} Jonathan K. Pritchard^{1,2,4,5†}

Histone modifications are important markers of function and chromatin state, yet the DNA sequence elements that direct them to specific genomic locations are poorly understood. Here, we identify hundreds of quantitative trait loci, genome-wide, that affect histone modification or RNA polymerase II (Pol II) occupancy in Yoruba lymphoblastoid cell lines (LCLs). In many cases, the same variant is associated with quantitative changes in multiple histone marks and Pol II, as well as in deoxyribonuclease I sensitivity and nucleosome positioning. Transcription factor binding site polymorphisms are correlated overall with differences in local histone modification, and we identify specific transcription factors whose binding leads to histone modification in LCLs. Furthermore, variants that affect chromatin at distal regulatory sites frequently also direct changes in chromatin and gene expression at associated promoters.

Variation at noncoding regulatory sequences contributes to the genetics of complex traits (1–3), yet we still have limited understanding of the primary mechanisms by which they act. One possibility is that regulatory variants affect histone modifications that have downstream consequences on chromatin remodeling or transcription (4). There are many possible post-translational modifications of histones (i.e., “histone marks”) (4), and sets of these co-occur in distinct chromatin states (5–9), are associated with functional elements (2, 10, 11), and are sensitive indicators of changes in gene regulation (9, 12). However, we still do not know whether histone modifications are generally a cause or a consequence of gene regulation or which DNA elements direct cell type–appropriate histone marking (7, 13). Thus, studies of genetic variants that disrupt transcription factor binding sites (TFBSs) may illuminate whether histone modifications enable transcription factor binding or whether the binding of transcription factors results in histone modification.

We performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) for RNA polymerase II (Pol II) and four posttranslational modifications of histone H3 (H3K4me1, H3K4me3, H3K27ac, and H3K27me3) in 10 unrelated Yoruba lymphoblastoid cell lines (LCLs). H3K4me3 (trimethylation of lysine 4) is primarily associated with active promoters; H3K4me1 (monomethylation of lysine 4) is associated with active chromatin outside of promoters (e.g., enhancers); H3K27ac (acetylation of lysine 27) is associated with both active promoters and enhancers (6, 14); and H3K27me3 (trimethylation of lysine 27) is associated with silencing by the polycomb repressive complex 2 (PRC2) (15, 16). We mapped the ChIP-seq reads to the human genome, controlling for mapping biases introduced by polymorphic sites (17). Comparisons with ENCODE (1) showed consistent distributions of each mark (fig. S1).

To identify genetic associations with histone marks and Pol II, we developed a “combined haplotype test” that uses both read depth and allelic imbalance to enable mapping of cis–quantitative trait loci (QTLs) with small sample sizes (17). We applied the combined haplotype test to hundreds of thousands of polymorphic sites with sufficient read depth (i.e., sites within ChIP-seq peaks) and identified more than 1200 histone mark and Pol II QTLs at a false discovery rate (FDR) of 20% (Fig. 1, A and B, and fig. S3). After merging overlapping regions, we identified a total of 27 distinct QTLs for H3K4me1, 469 for

H3K4me3, 730 for H3K27ac, 118 for Pol II, and 2 for H3K27me3 (which tends not to fall into strong peaks) (table S2). At an FDR threshold of 10%, we identified 582 distinct histone mark and Pol II QTLs (table S2). In principle, some of these signals might be due to imprinting (8) or random allelic inactivation; however, several lines of evidence indicate that most of the regions that we identify are conventional QTLs (supplemental text).

Many of the histone mark QTLs overlap previously identified QTLs for deoxyribonuclease (DNase I) sensitivity (denoted “dsQTLs”) (18). DNase I sensitivity is an indicator of open chromatin, and DNase I hypersensitive sites (DHSs) typically mark active regulatory regions that are associated with active histone marks and transcription factor binding (19). Indeed, we found an enrichment of low *P* values when testing for QTL associations with Pol II and all four histone marks at dsQTLs, compared to the genome-wide set of tested single-nucleotide polymorphisms (SNPs) (Fig. 1, A and B, and fig. S3). Nevertheless, although most histone mark and Pol II QTLs are within 1 kb of a DHS (table S5), many are far from known dsQTLs (fig. S8). This suggests that histone modifications may provide more power to detect differences in chromatin state beyond that of DNase I sensitivity.

We plotted aggregate ChIP-seq read depth around DHSs associated with dsQTLs (Fig. 2), grouping read counts according to whether an individual carries the genotype associated with high, medium, or low sensitivity at a dsQTL. Most of the dsQTLs lie outside promoters, and the average histone mark read depths at dsQTL DHSs follow qualitative expectations for distal enhancers (6), with higher levels of H3K4me1 and lower levels of H3K4me3 and Pol II as compared to promoters. High-sensitivity genotypes tend to have reduced nucleosome occupancy within the DHS (20); higher levels of transcription factor binding (18); higher levels of the active marks H3K4me1, H3K4me3, and H3K27ac; and higher Pol II occupancy. The relation between DNase I and the repressive mark H3K27me3 is more complicated, as we find both positive and negative associations. We find no opposite-direction effects between DNase I and either H3K4me1, H3K4me3, or H3K27ac (fig. S5).

At expression QTLs (eQTLs) (21), we stratified the samples by the genotype of the most significant eQTL SNP and found overall patterns similar to those at dsQTLs (Fig. 2). Individuals who are homozygous for the high-expression genotype generally have higher levels of DNase I sensitivity, H3K4me3, H3K27ac, and Pol II

¹Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA. ²Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA. ³Committee on Genetics, Genomics and Systems Biology, University of Chicago, Chicago, IL 60637, USA. ⁴Department of Genetics, Stanford University, Stanford, CA 94305, USA. ⁵Department of Biology, Stanford University, Stanford, CA 94305, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: gilad@uchicago.edu (Y.G.); pritch@stanford.edu (J.K.P.)

occupancy at transcription start sites (TSSs). The repressive H3K27me3 mark shows the opposite trend and is highest in the low-expression genotype class.

We estimated the correlation of allele-specific changes across pairs of data types, while accounting for the sampling variance at individual sites (17). The allelic imbalances for features associated with active regions—DNase I, Pol II, H3K4me1, H3K4me3, and H3K27ac—are all highly positively correlated across 2-kb windows centered at dsQTL DHSs (Fig. 1C). In particular, the strong correlation in H3K4me3 and H3K27ac allelic imbalances indicates that these modifications are functionally linked and often depend on the same genetic elements.

To test the hypothesis that histone modification is directed by sequence-specific transcription factors, we developed a statistical method to evaluate whether polymorphisms in TFBSs are associated with allelic imbalance in histone marks or Pol II (17). This method can infer causation because it is likely that these polymorphisms affect transcription factor binding. We identified 11,437 high-confidence TFBSs (22) that contain sequence polymorphisms in the 10 individuals that we studied. For each TFBS polymorphism, we computed the difference in the transcription factor position weight matrix (PWM) score between the two alleles (Δ PWM) and looked for associations between Δ PWM and allelic imbalance of ChIP-seq reads. The associations are positive and highly significant for the activating histone marks and Pol II [$P < 10^{-5}$ for all marks by likelihood ratio test (LRT)] and are significantly negative for H3K27me3 ($P = 0.028$ by LRT; Fig. 3B). As Δ PWM is positively correlated with transcription factor occupancy (18, 23) (fig. S11), these results suggest that increased transcription factor occupancy generally increases levels of nearby activating histone marks and lowers the levels of H3K27me3.

To identify specific transcription factors that direct histone marking, we grouped factors into clusters on the basis of sequence motifs and DNase I footprint similarity and tested TFBSs from each cluster for association between Δ PWM and allelic imbalance in the ChIP-seq reads. Out of the 39 clusters that have a sufficient number of polymorphic TFBSs to be testable, 11 have a significant association (FDR = 10% by LRT) with at least one histone mark (Fig. 3B). Most transcription factor clusters have positive associations with activating marks and negative (or nonsignificant) associations with H3K27me3. The transcriptional repressor NRSF (also called REST) is a prominent exception and has a positive association with H3K27me3 (Fig. 3A). NRSF directs PRC2-mediated gene silencing and H3K27me3 deposition during neuronal cell differentiation (24), and our results indicate that this factor may also be important for H3K27me3 deposition in lymphoblasts. These results demonstrate that transcription factor binding is often the first step in a series of events that leads to histone

modification, although they do not exclude the possibility that other factors may also have important causal roles.

Because dsQTLs are frequently also eQTLs (18), we used dsQTLs that are eQTLs (dsQTL-eQTLs) to assign DHSs to TSSs. We classified dsQTL-eQTLs as “activating” if the high-DNase I sensitivity allele was also the high-gene expression allele, and as “repressing” otherwise (Fig. 4A).

We confirmed that most activating dsQTL-eQTLs are true joint associations (as opposed to independent QTLs in linkage disequilibrium), but we discarded the repressive dsQTLs because only a small number had lower P values than expected by chance (fig. S10). We only used dsQTL-eQTLs where the associated DHS was at least 5 kb away from the associated TSS, so the regions are likely to be functionally distinct.

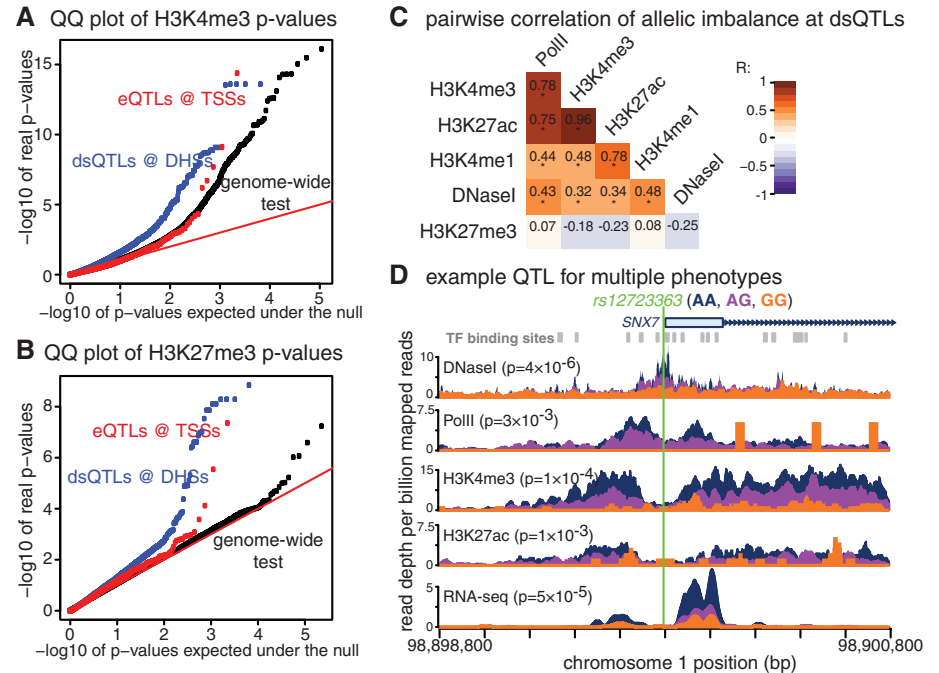


Fig. 1. Identification of histone mark and RNA polymerase II QTLs. (A) Quantile-quantile plot for H3K4me3 comparing expected to observed $-\log_{10} P$ values from the combined haplotype test. (B) Quantile-quantile plot for H3K27me3. (C) Correlation in allelic imbalance between data types at dsQTLs ($*P < 10^{-3}$ by likelihood ratio test). (D) An example of a QTL for multiple molecular phenotypes including DNase I sensitivity, gene expression, H3K4me3, H3K27ac, and Pol II levels. The tracks are colored by the genotype of the SNP rs12723363. P values were computed with the combined haplotype test, except for DNase I and RNA-seq, where a linear model (t test) was used.

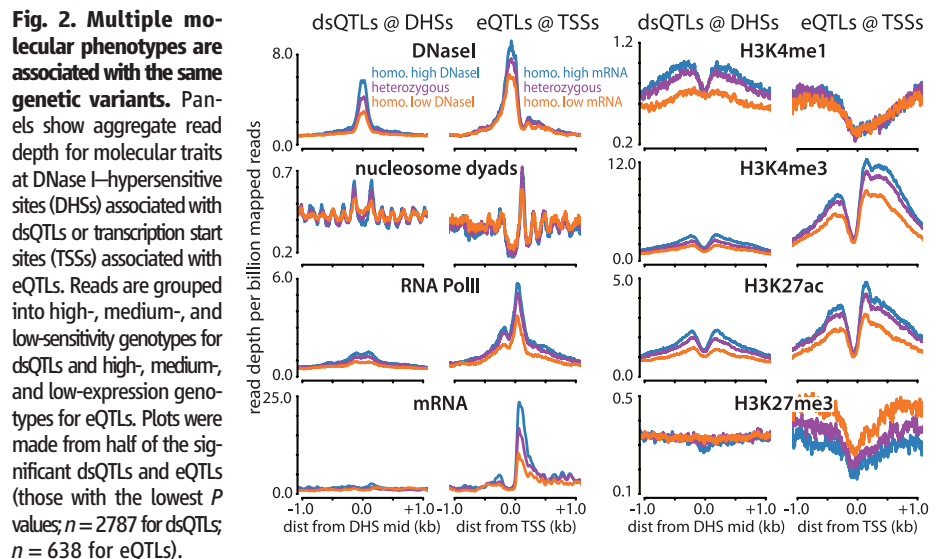


Fig. 2. Multiple molecular phenotypes are associated with the same genetic variants. Panels show aggregate read depth for molecular traits at DNase I-hypersensitive sites (DHSs) associated with dsQTLs or transcription start sites (TSSs) associated with eQTLs. Reads are grouped into high-, medium-, and low-sensitivity genotypes for dsQTLs and high-, medium-, and low-expression genotypes for eQTLs. Plots were made from half of the significant dsQTLs and eQTLs (those with the lowest P values; $n = 2787$ for dsQTLs; $n = 638$ for eQTLs).

For each dsQTL-eQTL pair, we estimated average allelic imbalance in histone marks and Pol II after polarizing genotypes by DNase I sensitivity at the associated DHS. At activating DHSs, the allelic imbalance is positive (in the same direction as DNase I sensitivity at the DHS) for the three activating histone marks and Pol II and

is negative for H3K27me3 (Fig. 4A). The same pattern is present at the associated TSSs, which demonstrates that polymorphisms can jointly affect chromatin state at distal enhancers and at promoters, perhaps via chromatin looping interactions (25). We found that for several of the dsQTL-eQTLs, the SNP that is most significant-

ly associated with DNase I sensitivity is located in a binding site for a known transcription factor (Fig. 4B).

In summary, our study allowed us to link genetic variation in a human population to variation in chromatin state. We identified QTLs associated with histone modification and Pol II binding that are enriched at both dsQTLs and eQTLs, and we found that single genetic variants may affect multiple aspects of chromatin state, including histone modification, DNase I sensitivity, and nucleosome positioning. In some cases, polymorphisms in TFBSs are causally responsible for differences in histone marking, and we have identified several specific transcription factors that are key regulators of histone marking in LCLs, an important step toward understanding how chromatin state is encoded by the genome.

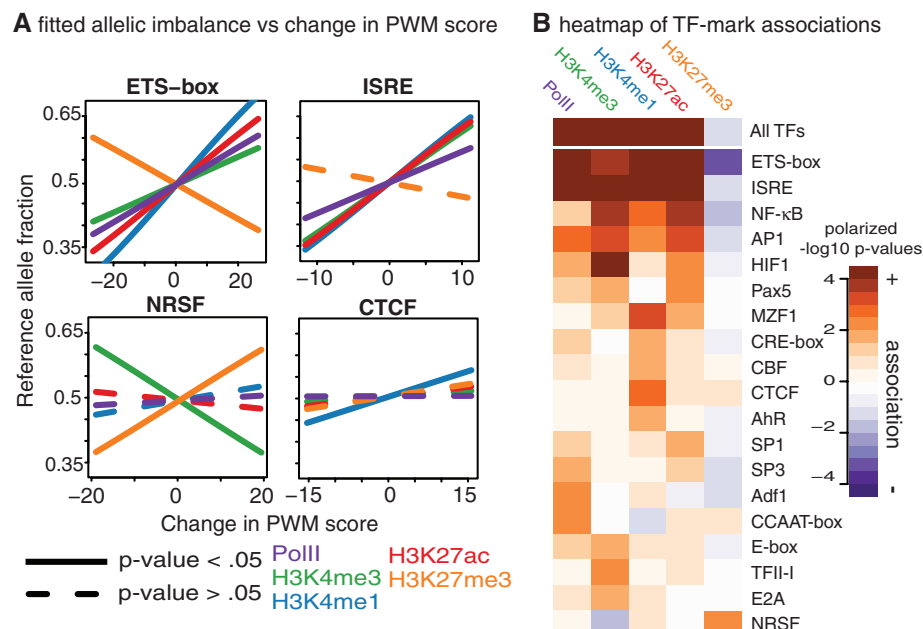


Fig. 3. Polymorphisms in transcription factor binding sites affect local histone modification. (A) Examples of transcription factor polymorphisms associated with histone marks or Pol II. Each plot shows the estimated relationship between difference in the transcription factor position weight matrix score between alleles (Δ PWM) and allelic imbalance (17). (B) Heatmap showing significance and direction of association between Δ PWM and allelic imbalance of histone marks or Pol II. Only transcription factor clusters with at least one nominally significant association are shown.

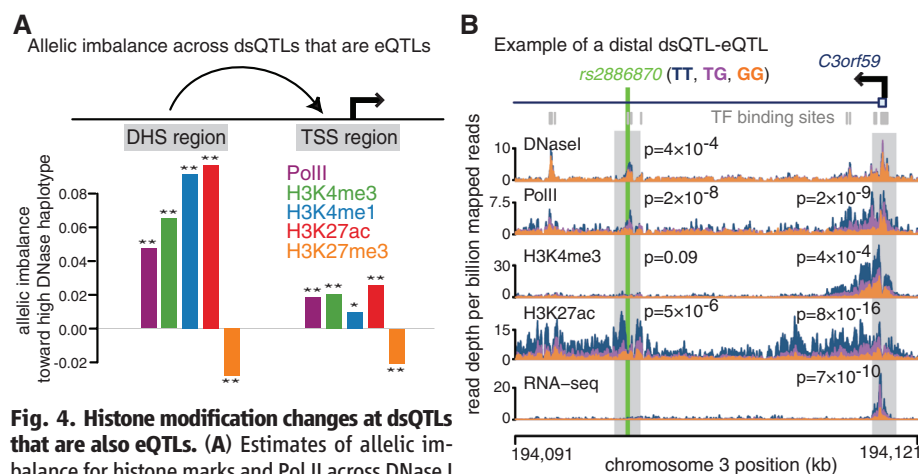


Fig. 4. Histone modification changes at dsQTLs that are also eQTLs. (A) Estimates of allelic imbalance for histone marks and Pol II across DNase I hypersensitive sites (DHSs; $n = 239$) and transcription start sites (TSSs; $n = 246$) from joint dsQTL-eQTLs (17). (*) and (**) indicate that allelic imbalance is significantly different from 0 with $P < 0.05$ and $P < 0.01$, respectively (by likelihood ratio test). (B) An example of a dsQTL-eQTL. The SNP rs2886870 disrupts a NF- κ B binding site and is significantly associated with local DNase sensitivity, H3K27ac, and Pol II levels. The SNP is also significantly associated with gene expression, H3K27ac, H3K4me3, and Pol II levels at a distal promoter (>18 kb away). P values are from the combined haplotype test, except for DNase I and RNA-seq, where linear regression and t tests were used. Read depth tracks are aggregated and colored by the genotype of rs2886870.

References and Notes

1. ENCODE Project Consortium, *Nature* **489**, 57–74 (2012).
2. J. Ernst et al., *Nature* **473**, 43–49 (2011).
3. M. A. Schaub, A. P. Boyle, A. Kundaje, S. Batzoglou, M. Snyder, *Genome Res.* **22**, 1748–1759 (2012).
4. T. Kouzarides, *Cell* **128**, 693–705 (2007).
5. B. E. Bernstein et al., *Cell* **125**, 315–326 (2006).
6. N. D. Heintzman et al., *Nature* **459**, 108–112 (2009).
7. T. Jenuwein, C. D. Allis, *Science* **293**, 1074–1080 (2001).
8. T. S. Mikkelsen et al., *Nature* **448**, 553–560 (2007).
9. A. Rada-Iglesias et al., *Nature* **470**, 279–283 (2011).
10. M. M. Hoffman et al., *Nat. Methods* **9**, 473–476 (2012).
11. P. V. Kharchenko et al., *Nature* **471**, 480–485 (2011).
12. J. A. Wamstad et al., *Cell* **151**, 206–220 (2012).
13. S. Henikoff, A. Shilatifard, *Trends Genet.* **27**, 389–396 (2011).
14. A. Barski et al., *Cell* **129**, 823–837 (2007).
15. B. Czermin et al., *Cell* **111**, 185–196 (2002).
16. J. Müller et al., *Cell* **111**, 197–208 (2002).
17. See supplementary materials and methods on Science Online.
18. J. F. Degner et al., *Nature* **482**, 390–394 (2012).
19. A. P. Boyle et al., *Cell* **132**, 311–322 (2008).
20. D. J. Gaffney et al., *PLOS Genet.* **8**, e1003036 (2012).
21. J. K. Pickrell et al., *Nature* **464**, 768–772 (2010).
22. R. Pique-Regi et al., *Genome Res.* **21**, 447–455 (2011).
23. T. E. Reddy et al., *Genome Res.* **22**, 860–869 (2012).
24. P. Arnold et al., *Genome Res.* **23**, 60–73 (2013).
25. A. Sanyal, B. R. Lajoie, G. Jain, J. Dekker, *Nature* **489**, 109–113 (2012).

Acknowledgments: We thank A. Ruthenberg, B. Howie, and members of the Pritchard, Przeworski, Stephens, and Gilad laboratories for discussions and three anonymous reviewers for comments. This work was supported by grants from the NIH (HG007036, HG006123, GM007197 and MH084703), by an NSF predoctoral award (DGE-0638477 to B.v.d.G.), and by the Howard Hughes Medical Institute. ChIP-seq data have been deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession GSE47991. RNA-seq, DNase-seq, and MNase-seq data were previously deposited with accessions GSE19480, GSE31388, and GSE36979. J.K.P. is on the scientific advisory boards for 23andMe and DNANexus with stock options.

Supplementary Materials

www.sciencemag.org/content/342/6159/747/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S11
Tables S1 to S5
References (26–43)

26 June 2013; accepted 12 September 2013
Published online 16 October 2013;
10.1126/science.1242429

Extensive Variation in Chromatin States Across Humans

Maya Kasowski,^{1,2*} Sofia Kyriazopoulou-Panagiotopoulou,^{3*} Fabian Grubert,^{1*} Judith B. Zaugg,^{1*} Anshul Kundaje,^{1,3,4,5*} Yuling Liu,⁸ Alan P. Boyle,¹ Qiangfeng Cliff Zhang,¹ Fouad Zakharia,¹ Damek V. Spacek,¹ Jingjing Li,¹ Dan Xie,¹ Anthony Olarerin-George,⁶ Lars M. Steinmetz,^{1,7} John B. Hogenesch,⁶ Manolis Kellis,^{4,5} Serafim Batzoglou,³ Michael Snyder^{1†}

The majority of disease-associated variants lie outside protein-coding regions, suggesting a link between variation in regulatory regions and disease predisposition. We studied differences in chromatin states using five histone modifications, cohesin, and CTCF in lymphoblastoid lines from 19 individuals of diverse ancestry. We found extensive signal variation in regulatory regions, which often switch between active and repressed states across individuals. Enhancer activity is particularly diverse among individuals, whereas gene expression remains relatively stable. Chromatin variability shows genetic inheritance in trios, correlates with genetic variation and population divergence, and is associated with disruptions of transcription factor binding motifs. Overall, our results provide insights into chromatin variation among humans.

Association and gene expression studies have linked disease predisposition to specific alleles (1–3) and identified intermediate molecular phenotypes that may be

responsible for organismal differences (4–7). However, the underlying mechanisms by which genetic variation drives either disease or expression differences remain poorly understood. Interindividual variability has been reported for transcription factor (TF) binding (8–10) and deoxyribonuclease I (DNase I) accessibility (11, 12). However, TF studies assay a very small fraction of regulatory elements, whereas DNase I hypersensitivity does not distinguish between different types of regulatory elements (e.g., enhancers versus promoters), is biased toward active elements, and provides little information on domain-level features (e.g., Polycomb-repressed domains).

To further characterize human variation in diverse types of regulatory elements, we studied the chromatin state of lymphoblastoid cell lines (LCLs)

derived from 19 individuals: five European (CEU), seven Yoruban (YRI), and two Asian individuals from the 1000 Genomes Project (including two mother-father-daughter trios) (13), an additional Caucasian individual (14), and four deeply sequenced individuals from the San population (15) (table S1). We used RNA sequencing (RNA-seq) to measure expression and chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) to map five histone modifications (H3K27ac, H3K4me1, H3K4me3, H3K36me3, and H3K27me3) and two general factors (CTCF and SA1, a subunit of cohesin) (figs. S1 and S2 and tables S1 to S4). ChIP-seq reads were mapped to each line's phased genome to reduce mapping biases.

To systematically identify variable regions across individuals, we used analysis of variance (ANOVA) as well as DESeq (figs. S3 and S4) (16). Active chromatin marks H3K27ac, H3K4me1, and H3K4me3, and the repressive mark H3K27me3 show the highest fraction of variable regions, in contrast to gene-body marks and RNA expression levels (Fig. 1A and fig. S4).

We additionally used ChromHMM (17) to segment the genome of each individual into 15 chromatin states based on the combinatorial patterns of the chromatin marks and CTCF (Fig. 1, B and C, figs. S5 to S8, and table S5). We found that enhancer states exhibit the most variability (fig. S9), with bivalent (poised) enhancers having the highest fraction of individual-specific regions, followed by weak enhancers, followed by strong active enhancers. Similarly, bivalent promoters are more variable than active promoters and strongly transcribed states.

The variability of chromatin marks is often dependent on functional context defined by combinatorial chromatin patterns. H3K27ac and H3K4me3 show significantly higher variability at enhancers

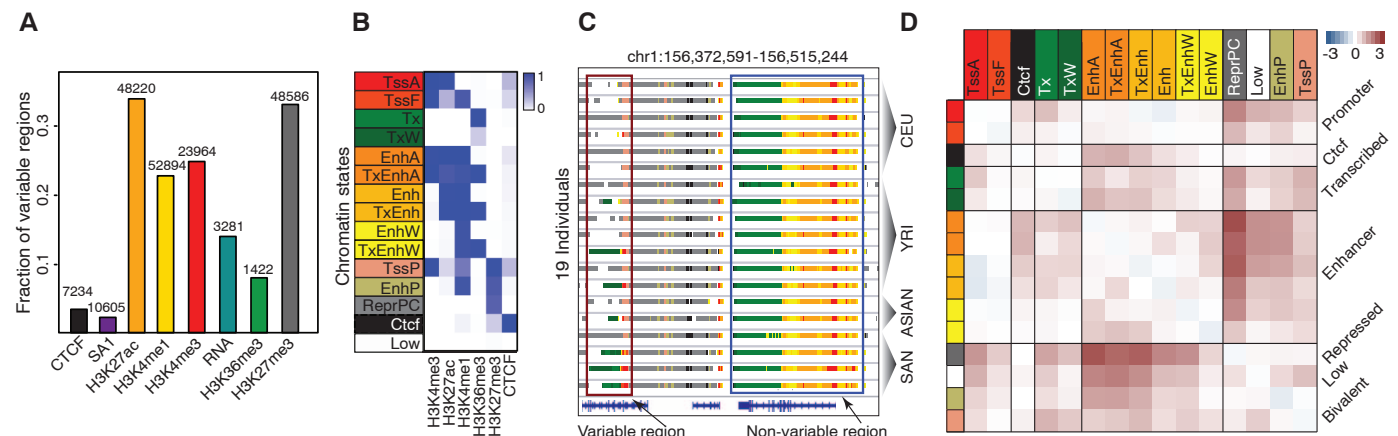


Fig. 1. Variation in chromatin, factors, and expression across individuals. (A) Number and fraction of enriched regions (for chromatin marks and factors) and expressed genes (for RNA) that are variable across individuals. (B) Composition (emission probability) of five chromatin marks and CTCF in 15 chromatin states: TssA (active promoters), TssF (flanking active promoters), Tx (strong transcription), TxW (weak transcription), EnhA (active enhancers with H3K4me3), TxEnhA (active enhancers in transcribed regions), Enh (active enhancers without H3K4me3), TxEnh (active enhancers without

H3K4me3 in transcribed regions), EnhW (weak enhancers), TxEnhW (weak enhancers in transcribed regions), TssP (poised promoters), EnhP (poised enhancers), ReprPC (Polycomb repressed), Ctcf (CTCF enriched regions), and Low (low signal). (C) Examples of a nonvariable and a variable region. Coordinates are in build hg19 of the human reference sequence. State colors are as in (B). (D) log₁₀ ratio of the observed probability that a region switches from one state (row) to another (column) in any pair of individuals relative to background switching across pairs of replicates.

compared with promoters (fig. S10). This could explain the apparent discrepancy between the high variability of H3K4me3 and the low variability of expression and gene body marks (Fig. 1A). Furthermore, the repressive mark H3K27me3 is significantly more variable when co-enriched with other marks in bivalent states—such as poised enhancers and poised promoters—than in stable Polycomb-repressed domains (Fig. 1B and fig. S10).

Investigating the dynamics of chromatin state conversions among individuals, we found that most significant state switches are between active states, such as enhancers, promoters, or transcribed regions, and repressed or weakly active states (Fig. 1D and fig. S11). Although changes in ac-

tivity are common, switching between enhancers and core promoter states is rare, highlighting that these are distinct types of regulatory elements.

We examined the effects of enhancer variability on gene expression and found no significant difference in expression variability between genes with one variable enhancer and those lacking variable enhancers. However, there is a significant increase in expression variability when more than 60% of the gene's enhancers vary (fig. S12A; Wilcoxon's rank sum test, $P < 0.05$), indicating that changes in multiple enhancers are often required to alter gene expression. We also found that 74% of nonvariable genes and 99% of variable genes are associated with at least one var-

iable enhancer (24-fold enrichment, Fisher's exact test, $P < 2.2 \times 10^{-16}$) (15, 18) and that enhancer-gene expression correlations are stronger for genes with a single enhancer than for genes linked to multiple enhancers (fig. S12B; Wilcoxon, $P = 7 \times 10^{-5}$). Thus, a substantial fraction of the enhancers that are variable across individuals do not result in detectable differences in gene expression, suggesting that compensatory regulatory effects, enhancer redundancy, subtle gene expression variation, or nonconsequential enhancer variation exist under the experimental conditions examined.

Variable regions are enriched in single-nucleotide polymorphisms (SNPs) relative to nonvariable regions (2.8-fold; $P < 2.2 \times 10^{-16}$; Fisher's exact test), with an increased number of SNPs associated with higher variability (fig. S13). Signal variability also increases with nucleotide diversity ($P < 1 \times 10^{-10}$; Wilcoxon test) (15). Consistently, the correlation between genotype and signal is stronger for variable than nonvariable H3K27ac peaks ($P < 1 \times 10^{-15}$; Wilcoxon test) (Fig. 2A). Nonvariable H3K4me1 and H3K27ac peaks have suppressed derived allele frequencies in both the Yoruban and Caucasian populations ($P < 2 \times 10^{-5}$; Wilcoxon test) and increased conservation scores ($P < 0.005$; binomial test) (15) compared with variable regions, suggesting stronger negative selection in nonvariable regions. Also, the fraction of heterozygous SNPs with allele-specific signal is highest for the active marks H3K27ac, H3K4me1, and H3K4me3 (fig. S14A), which is in agreement with cis effects on the variability of these marks. Finally, rare variants (allele frequency < 0.01 in the 1000 Genomes Project) are enriched in variable H3K27ac regions compared with nonvariable regions ($P < 2.5 \times 10^{-14}$; two-sample t test), indicating that rare variants may underlie enhancer variation.

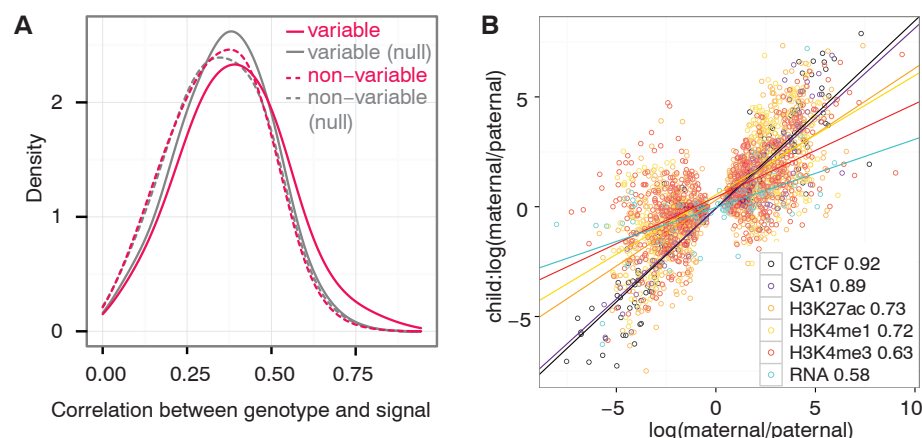


Fig. 2. Genetic basis of chromatin variation. (A) Spearman correlation between genotype and signal at variable and nonvariable H3K27ac regions after correcting for differences in length and signal strength. For the null sets, we shuffled the signal. (B) Correlation of allelic biases between the parents and the daughter of the YRI trio at allele-specific SNPs of the daughter that are homozygous in both parents (Pearson correlation coefficients are in the legend; linear fits are shown as lines). Only marks with at least 50 SNPs are shown.

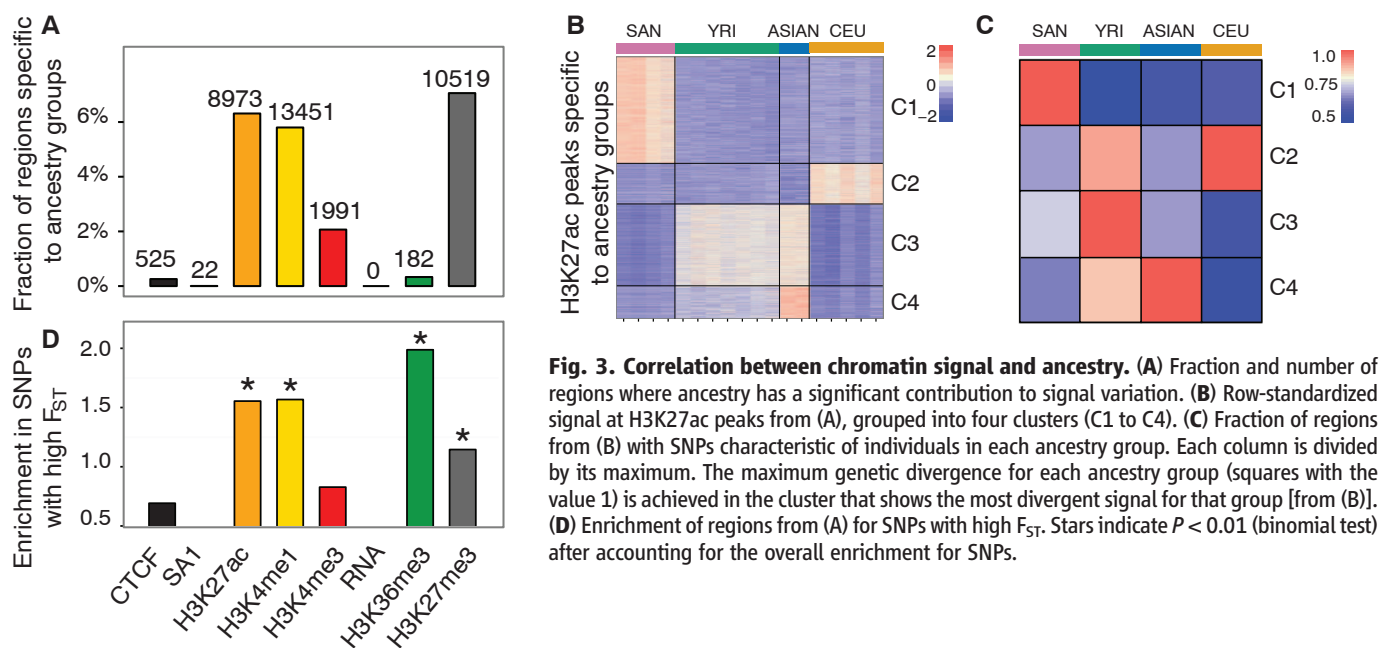


Fig. 3. Correlation between chromatin signal and ancestry. (A) Fraction and number of regions where ancestry has a significant contribution to signal variation. (B) Row-standardized signal at H3K27ac peaks from (A), grouped into four clusters (C1 to C4). (C) Fraction of regions from (B) with SNPs characteristic of individuals in each ancestry group. Each column is divided by its maximum. The maximum genetic divergence for each ancestry group (squares with the value 1) is achieved in the cluster that shows the most divergent signal for that group [from (B)]. (D) Enrichment of regions from (A) for SNPs with high F_{ST} . Stars indicate $P < 0.01$ (binomial test) after accounting for the overall enrichment for SNPs.

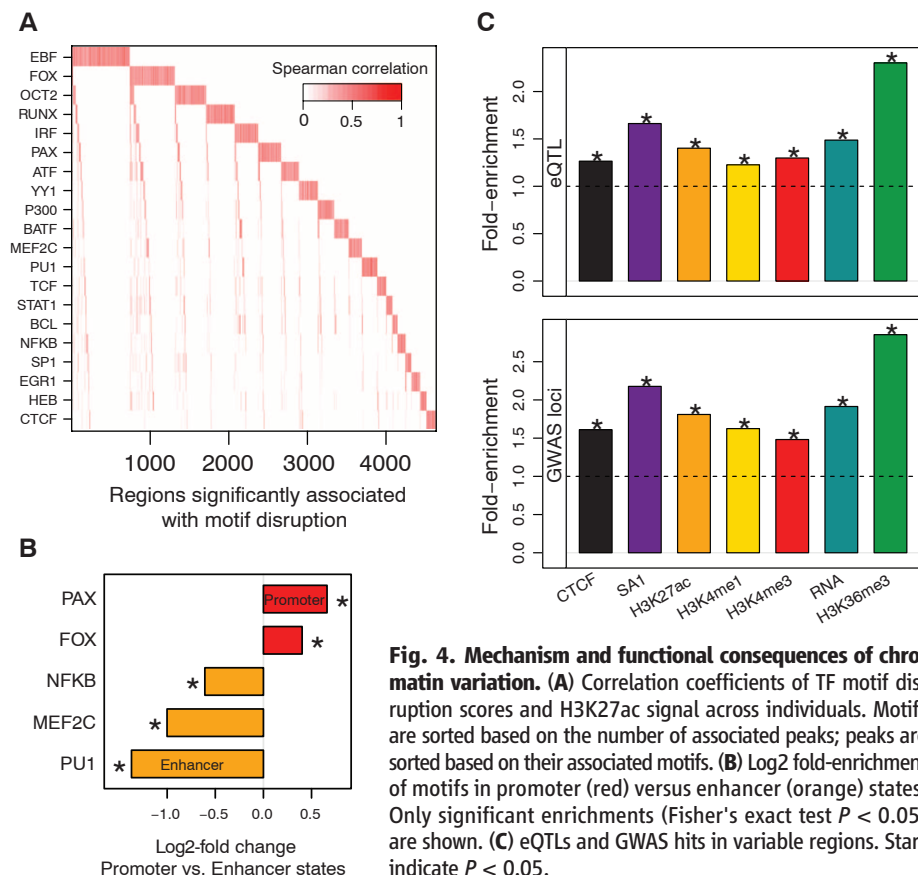


Fig. 4. Mechanism and functional consequences of chromatin variation. (A) Correlation coefficients of TF motif disruption scores and H3K27ac signal across individuals. Motifs are sorted based on the number of associated peaks; peaks are sorted based on their associated motifs. (B) Log₂ fold-enrichment of motifs in promoter (red) versus enhancer (orange) states. Only significant enrichments (Fisher's exact test $P < 0.05$) are shown. (C) eQTLs and GWAS hits in variable regions. Stars indicate $P < 0.05$.

We observed strong correlation of allele-specific signal between daughters and parents, especially for CTCF, SA1, and the enhancer and promoter marks, which suggests that the patterns of chromatin modifications and TF binding are heritable (Fig. 2B and fig. S14B). For the majority of marks, more than 75% of sites agree in the direction of allelic bias between daughters and parents (fig. S14, C and D). Gene expression is less heritable (Fig. 2B), in agreement with previous studies (19).

Next, we analyzed variation across individuals grouped by ancestry. For all marks, ancestry explains less than 20% of the variance at a majority of regions (fig. S15). The enhancer marks H3K27ac and H3K4me1 have the largest fraction of regions that discriminate ancestry groups [F -test corrected $P < 0.01$ (15)] (Fig. 3, A and B, and figs. S16 and S17, A and B), with signal divergence often correlating with genetic divergence (Fig. 3C). The expression of genes overlapping these regions shows a similar but weaker pattern (fig. S17C), suggesting that the impact of genetic variation at regulatory elements may be diluted at the level of downstream expression. Regions with divergent signal across ancestry groups are enriched for SNPs

compared with other regions for the same marks (binomial $P = 2 \times 10^{-58}$ to 1.8×10^{-5} for all marks) (fig. S17D). They are also enriched for SNPs with high fixation index (F_{ST}) (20), a measure of genetic divergence across populations (binomial $P = 1 \times 10^{-7}$ to 0.01) (Fig. 3D). Although the observed signal patterns may not generalize to larger samples, they establish a link between chromatin variation and genetic divergence.

One possible mechanism through which genetic variability leads to chromatin variation is the disruption of TF binding (8, 12). We found that variable regions of active chromatin marks are enriched for motif-disrupting SNPs (1.3- to 2.3-fold; Fisher's exact test $P < 5.1 \times 10^{-46}$) (fig. S18A). Of the variable H3K27ac regions overlapping Encyclopedia of DNA Elements TF binding sites in GM12878 (21), 32% show significant associations between signal differences and motif disruptions (fig. S18B) (15). The most frequent motif disruptions involve cell-type-specific regulatory factors (Fig. 4A and fig. S19), some of which are differentially associated with H3K27ac variation at enhancer and promoter states (Fig. 4B). Finally, variable regions and allele-specific SNPs are enriched for DNase I sensitivity quantitative

trait loci (dsQTLs), expression QTLs (eQTLs), and genome-wide association studies (GWAS) SNPs, providing further evidence of the functional implications of chromatin variability (Fig. 4C and fig. S20).

In summary, enhancers are highly variable and may contribute to phenotypic differences between individuals and ancestral groups through heritable variation in histone modifications arising from SNPs in TF binding sites.

References and Notes

- Wellcome Trust Case Control Consortium, *Nature* **447**, 661–678 (2007).
- M. I. McCarthy *et al.*, *Nat. Rev. Genet.* **9**, 356–369 (2008).
- L. A. Hindorf *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 9362–9367 (2009).
- M. Morley *et al.*, *Nature* **430**, 743–747 (2004).
- B. E. Stranger *et al.*, *Nat. Genet.* **39**, 1217–1224 (2007).
- A. L. Dixon *et al.*, *Nat. Genet.* **39**, 1202–1207 (2007).
- J. K. Pickrell *et al.*, *Nature* **464**, 768–772 (2010).
- M. Kasowski *et al.*, *Science* **328**, 232–235 (2010).
- A. R. Borneman *et al.*, *Science* **317**, 815–819 (2007).
- W. Zheng, H. Zhao, E. Mancera, L. M. Steinmetz, M. Snyder, *Nature* **464**, 1187–1191 (2010).
- R. McDaniell *et al.*, *Science* **328**, 235–239 (2010).
- J. F. Degner *et al.*, *Nature* **482**, 390–394 (2012).
- G. R. Abecasis *et al.*, *Nature* **467**, 1061–1073 (2010).
- R. Chen *et al.*, *Cell* **148**, 1293–1307 (2012).
- Materials and methods and supporting data are available on Science Online.
- S. Anders, W. Huber, *Genome Biol.* **11**, R106 (2010).
- J. Ernst, M. Kellis, *Nat. Methods* **9**, 215–216 (2012).
- C. Y. McLean *et al.*, *Nat. Biotechnol.* **28**, 495–501 (2010).
- T. E. Reddy *et al.*, *Genome Res.* **22**, 860–869 (2012).
- S. Duan, W. Zhang, N. J. Cox, M. E. Dolan, *Bioinformatics* **3**, 139–141 (2008).
- M. B. Gerstein *et al.*, *Nature* **489**, 91–100 (2012).

Acknowledgments: Funded by grants from the NIH and Genetics Department, Stanford University, Vera Moulton Wall Center for Pulmonary Vascular Disease and NIH MSTP TG T32GM07205 (M.K.), the Siebel Scholars Foundation (S.-K.P.), the KAUST-Stanford Academic Excellence Alliance program (S.-K.P. and S.B.), the Swiss National Foundation, and the Janggen-Poehn Foundation (J.B.Z.). Data sets are available at the Gene Expression Omnibus (GEO) database with accession no. GSE50893. We thank S. Montgomery, W. Huber, C. Bustamante, H. Tang, S. Anders, G. Euskirchen, B. Altschuler, M. Eaton, and L. Ward. M.S. is a founder and member of the science advisory board for Personalis and a science advisory board member for Genapsys and Axiom. S.B. is a founder and advisor for DNAnexus and serves on the advisory boards of 23andMe and Eve Biomedical. Genotype calls and BAM files with mapped sequencing reads for the San individuals are available through a data access agreement for transfer of genetic data by contacting M.S.

Supplementary Materials

www.sciencemag.org/content/342/6159/750/suppl/DC1
Materials and Methods
Figs. S1 to S20
Tables S1 to S5
References (22–41)

27 June 2013; accepted 17 September 2013
Published online 17 October 2013;
10.1126/science.1242510



cell sciences
cytokine center



www.cellsciences.com

Buy one, get one free

Stock up now on select cytokines, growth factors and chemokines.

Order any two vials from this list for just \$235

Enter promo code CYT241 when you place your order on-line or mention the promo code to get your discount when calling our toll-free number 888-769-1246.

These recombinant proteins are low endotoxin, carrier-free, highly pure, biologically active and suitable for cell culture and animal studies. Get two of the same item or pick any two different items from the list.* See our web site for complete product specifications.

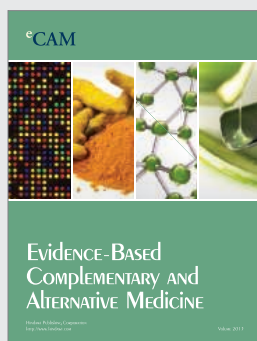
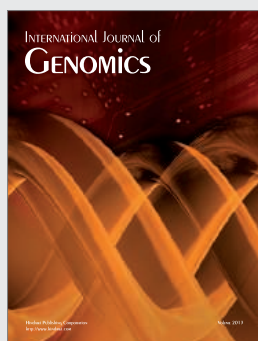
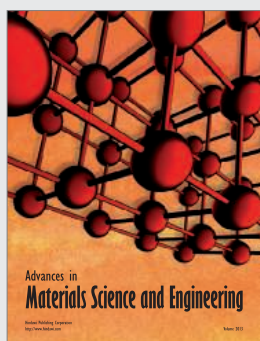
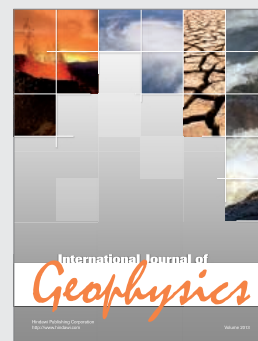
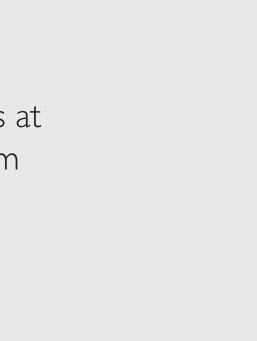
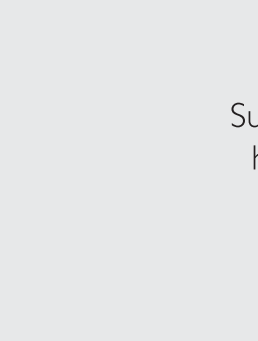
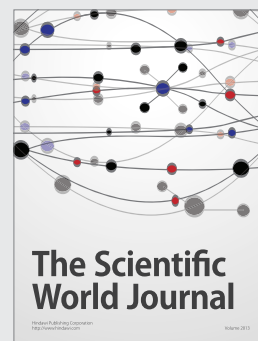
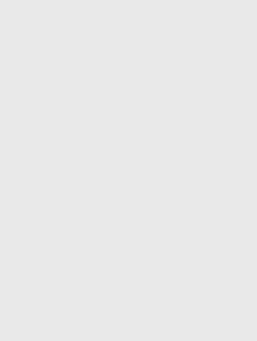
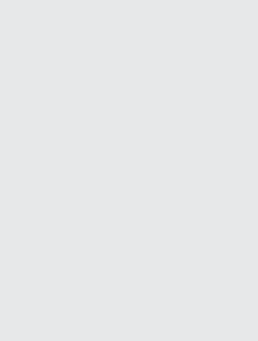
Browse our web site of recombinant proteins, including hundreds more cytokines, growth factors, chemokines and neurotrophins. Bulk quantities of these proteins are available at competitive pricing. Call or visit our web site for details. Cell Sciences also carries corresponding antibodies and ELISA kits.

Catalog No:	Item:	Size:
CRB100B	Recombinant Human BMP-2	10 µg
CRC400B	Recombinant Human CNTF	20 µg
CRF000B	Recombinant Human FGF-acidic/FGF1	50 µg
CRF001B	Recombinant Human FGF-basic/FGF2	50 µg
CRK300B	Recombinant Human FGF-7/KGF	10 µg
CRG300B	Recombinant Human G-CSF/CSF3	10 µg
CRG100B	Recombinant Human GM-CSF/CSF2	20 µg
CRG101B	Recombinant Mouse GM-CSF/CSF2	20 µg
CRG500B	Recombinant Human GRO-alpha/CXCL1	25 µg
CRG502B	Recombinant Rat GRO-alpha/KC/CXCL1	25 µg
CRI004B	Recombinant Human IFN-alpha 2b	100 µg
CRI000B	Recombinant Human IFN-gamma	100 µg
CRI001B	Recombinant Mouse IFN-gamma	100 µg
CRI002B	Recombinant Rat IFN-gamma	100 µg
CRI500B	Recombinant Human IGF-1	100 µg
CRI100B	Recombinant Human IL-2	50 µg
CRI153B	Recombinant Human IL-10	10 µg
CRI137B	Recombinant Human IL-15	10 µg
CRI162B	Recombinant Human IL-17	25 µg
CRI172B	Recombinant Human IL-21	10 µg
CRI225B	Recombinant Human IL-33	10 µg
CRM151B	Recombinant Human M-CSF/CSF1	10 µg
CRR000B	Recombinant Human RANTES/CCL5	20 µg
CRS000B	Recombinant Human SDF-1 alpha	10 µg
CRS002B	Recombinant Human SDF-1 beta	10 µg
CRT100B	Recombinant Human TNF-alpha	50 µg
CRT192B	Recombinant Mouse TNF-alpha	20 µg
CRV000B	Recombinant Human VEGF 165	10 µg
CRV014B	Recombinant Mouse VEGF 165	10 µg

* Good on orders in US and Canada only. This offer may not be combined with other offers or discounts. All products for research use only.

CELL SCIENCES INC • 480 NEPONSET STREET, BUILDING 12A, CANTON, MA 02021 • INFO@CELLSCIENCES.COM

TOLL FREE: (888) 769-1246 • TEL: (781) 828-0610 • FAX: (781) 828-0542 • WEB WWW.CELLSCIENCES.COM



Better results — on any sequencing platform

Get the most from your NGS

Discover new and innovative solutions,
dedicated for use with any NGS workflow

Streamline your next-generation sequencing (NGS) workflow and achieve high-quality results you can rely on.

- **Highly specific and selective nucleic acid purification and target enrichment**
- **Unbiased whole genome amplification from a single cell**
- **High DNA library yields using optimized workflows that allow ~50% time-savings**
- **Outstanding results on any sequencing platform**
- **Intuitive, knowledge-based data interpretation for deeper insight into NGS results**

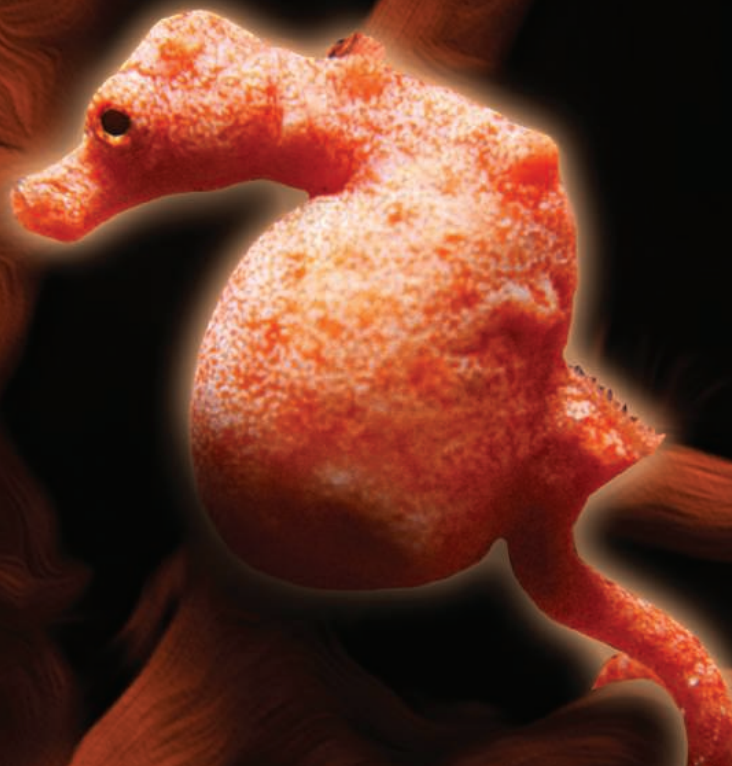
Visit www.qiagen.com/goto/NGS to learn more!



Sample & Assay Technologies

Enhanced BD Horizon™ Brilliant Violet™ Reagents

Get 30% off when you try the new, innovative formulation.



Revealing nature's secrets
with unprecedented clarity.

Now BD Horizon™ Brilliant Violet™ reagents have been enhanced for better performance.

BD Biosciences commitment to quality includes continual improvement of the flow cytometry tools we produce. Those efforts have yielded BD Horizon Brilliant Violet polymer conjugates that can more easily and precisely help you resolve rare and dim cell populations. Now accompanied by an innovative BD Biosciences proprietary staining buffer, the combined formulation resolves possible dye-to-dye interactions for consistently predictable results.



BD

Helping all people
live healthy lives

This pioneering polymer dye technology enables researchers to identify cell populations with lower receptor density than previously possible and resolve cell populations previously obscured. The complete portfolio of BD conjugated antibodies can be used to explore cellular features and characterize cells through surface and intracellular markers.

Take advantage of 30% savings for a limited time when you add BD Horizon Brilliant Violet reagents to your multicolor panel. Offer details, expanded tools, and information are available at bdbiosciences.com/go/brilliant.



He is using the
thermal cycler.



So is she.



So is he.

Introducing the ProFlex™ PCR System

Applied Biosystems®



3 different users. 3 different experiments. 3 different
times. All connected remotely to one high-performing
and flexible instrument built for today's PCR laboratory.

Buy one at lifetechnologies.com/proflexpcr

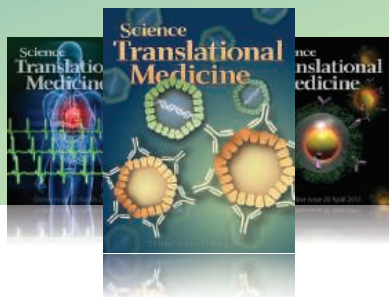
life
technologies™

For Research Use Only. Not for use in diagnostic procedures. ©2013 Life Technologies Corporation. All rights reserved. The trademarks mentioned herein are the property of Life Technologies Corporation and/or its affiliate(s) or their respective owners. C0113461 0513

Science Translational Medicine

Integrating Science,
Engineering,
and Medicine

Publishing results that
harness the basic sciences
to advance human health
in all areas of medicine



Submit your research

ScienceTranslationalMedicine.org

Recommend to your library

ScienceOnline.org/recommend



scitranslmededitors@aaas.org

AAAS Travels



GALAPAGOS ISLANDS

On board *National Geographic Endeavour*
July 25–August 3, 2014

There is something special about Galápagos... renowned for birdlife, marine wildlife, unusual plants, lava flows, and the tortoise population. Ship lecturers intrigue you while on shore walks, snorkeling with sea lions, or photography excursions, and much more! From \$5,990 + air.

**For a detailed brochure,
please call (800) 252-4910**

All prices are per person twin share + air



BETCHART EXPEDITIONS Inc.

17050 Montebello Rd, Cupertino, CA 95014

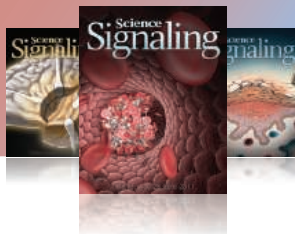
Email: AAASInfo@betchartexpeditions.com

www.betchartexpeditions.com



Science Signaling

Publishing key findings
in cell signaling
in all areas of biology



Submit your research

ScienceSignaling.org



sciencesignalingeditors@aaas.org

Easily
manage your
instrument use
and care online



Sign in to your account on
lifetechnologies.com* to:

- Access instrument service history
- Request service and get quotes
- Track warranty and contract status
- Check availability and schedule instrument use
- Discover many more benefits

No cost. No hassle. No worries.

Learn more at
[lifetechnologies.com/
easiertomanage](http://lifetechnologies.com/easiertomanage)

life
technologies™

*At this time, Instrument Management is available in the United States, Canada, Australia, New Zealand, and most countries in Europe.
©2013 Life Technologies Corporation. All rights reserved. C007223 0913

NEW NAME. NEW ADVANCES. THE METHODIST HOSPITAL IS NOW HOUSTON METHODIST HOSPITAL.

We've changed our name. The Methodist Hospital is now Houston Methodist Hospital. We're always moving forward, and we believe that our greatest advances are those that are just around the corner. So we continue to look toward the future, bringing the finest researchers and clinicians from around the world to Houston, to join us in the pursuit of better care and better cures. That's the difference between practicing medicine and leading it.

houstonmethodist.org

HOUSTON
MethodistSM
LEADING MEDICINE



You can help students reach new heights.

With nearly 100 volunteers spending a day a week in K-12 classrooms in the D.C. area, and a budding program in Seattle, AAAS is committed to working with scientists and engineers to make scientific literacy possible for all students.

Now, thanks to a generous donation from a AAAS Fellow, we are ready to grow these efforts into a nationwide network.

Toward that end, AAAS is announcing the pilot stage of the AAAS National STEM Volunteer Program.

In 2014, AAAS will award five seed grants of up to \$15,000 each to members working with non-profits in their community to bring scientists and engineers into local classrooms in an ongoing and meaningful way.

In addition to funding, AAAS will provide resources for developing, implementing, and sustaining the volunteer effort.

The deadline for submitting a letter of intent is November 18, 2013, and the full application deadline is December 20, 2013. All grants will be awarded by February 28, 2014.

To learn more, and to submit your application, visit:
<http://membercentral.aaas.org/volunteer/ANSVP>.

AAAS
 NATIONAL STEM
 VOLUNTEER PROGRAM

PROGRAMMABLE CIRCULATING BATHS

A line of highly sophisticated circulating water baths makes it simple to create and run multistep ramp and soak temperature programs as well as frequently used single-temperature testing protocols. Designed with the work habits and needs of laboratory personnel in mind, the Performance Programmable Circulating Baths feature an icon-driven touchscreen display that makes selecting and setting operational parameters simple and straightforward. A built-in electronic keypad speeds and simplifies the entry of text and numerical values. Available in both refrigerating/heating and heat-only models, the baths control water temperatures as broad as -40°C to $+200^{\circ}\text{C}$ with $\pm 0.005^{\circ}\text{C}$ stability and feature OpenMode programming, which places no restrictions on the number of time/temperature programs or steps that can be stored. Highly customizable, they feature a variety of user-settable operational parameters, including the size, style, and type of temperature information that is displayed as well as the menu/display language.

PolyScience

For info: 800-229-7569 | www.polyscience.com



FLAME BURNER

The innovative Fuego SCS Series provides maximum safety and highest convenience for all flame-related applications in the laboratory. The Fuego SCS offers more convenience through the new graphic display and comfort functions. The animated, self-explanatory, and wordless symbol display facilitates a rapid and easy selection of all functions. To assist the user the new comfort functions provide: zero-pressure shut off, gas consumption display, cooling reminder for inoculation loops, temperature regulation for heating media, user account selection, graphical installation instructions, and acoustic signals as operating aids. Fuego's streamlined design reduces air flow disturbances in laminar flow hoods. The Safety Control System (SCS) demonstrates state-of-the-art safety technology which constantly analyzes potential hazards and, if necessary, initiates safety measures, such as an interruption of the gas supply. Exceptional passive safety features include a residual heat display that will signal that the burner head is still hot in order to protect the user from burns.

WLD-TEC GmbH

For info: +49-551-793789 | www.wld-tec.com

PIPETTE FILLING AND DISPENSING

The PIPETBOY pro sets the benchmark for fast, accurate pipette filling and dispensing. The accuracy of pipetting depends on precise graduations on your serological pipette and on your ability to accurately match the liquid's meniscus with the graduation mark. The PIPETBOY pro enables the user to very accurately displace liquid in the pipette by applying variable finger pressure on its pipette triggers. To operate a PIPETBOY pro simply fully press the aspiration trigger to quickly draw liquid into the pipette until almost full, then gradually release the trigger to reduce speed and accurately match the meniscus to the graduation mark. Similarly, when dispensing, slightly press the trigger for drop-by-drop dispensing of liquid, or fully trigger the powerful pump motor for fast emptying of the pipette. Accurate and reliable results can be achieved quickly even by occasional users at pipetting speeds of up to 12 mL/sec.

Integra Biosciences

For info: +41-(0)-81-286-95-30 | www.integra-biosciences.com

MICROPLATES

The Krystal 2000 microplate range has been designed to address performance, limiting well-to-well crosstalk inherent in most assay plates. Unique individual clear cups molded into either a black or white polypropylene matrix eliminate crosstalk and improve the sensitivity, photometric accuracy, and repeatability of the bottom reading for absorbance, fluorescence, and luminescence measurements. The Krystal 2000 range is fully compatible with all commercially available plate readers, robotic sample processors, and automated liquid handling systems. Constructed in ultrapure-grade polystyrene, Krystal 2000 microplates are available in opaque white, solid black, and black and white combination formats. For sensitive fluorescence measurements the black plate provides the all-absorbing background needed to minimize background interference. The opaque white plate maximizes reflectivity enabling even weakly emitting luminescence assays to be routinely undertaken. The unique design also offers improved cell binding efficiency and allows the convenience of direct measurements on bottom reading spectrophotometers and inverted microscopes.

Porvair Sciences

For info: +44-(0)-1372-824290 | www.porvair-sciences.com

MICROCENTRIFUGES

Designed to meet the specific requirements of a wide range of research applications, Microfuge 20 and 20R microcentrifuges are efficient and easy to use. Paired with Microfuge series rotors, the compact instruments provide flexibility and reliable, precise performance while occupying a small footprint. Processes are performed seamlessly, and include nucleic acid and protein preparation; pelleting, extractions, purifications, concentrations, phase separations, and receptor binding; and rapid sedimentation of protein precipitates, particulates, and cell debris. Entry and recall of up to 10 user-defined programs is facilitated by an easy-to-use interface and saves setup time for common protocols. Increased energy efficiency lowers utility costs. Samples can be processed at speeds up to 15,000 rpm (20,627 x g) in the Microfuge 20 and in the 20R, a refrigerated version with a temperature range of -10°C to 40°C .

Beckman Coulter Life Sciences

For info: 800-742-2345 | www.beckmancoulter.com

Electronically submit your new product description or product literature information! Go to www.sciencemag.org/products/newproducts.dtl for more information.

Newly offered instrumentation, apparatus, and laboratory materials of interest to researchers in all disciplines in academic, industrial, and governmental organizations are featured in this space. Emphasis is given to purpose, chief characteristics, and availability of products and materials. Endorsement by Science or AAAS of any products or materials mentioned is not implied. Additional information may be obtained from the manufacturer or supplier.



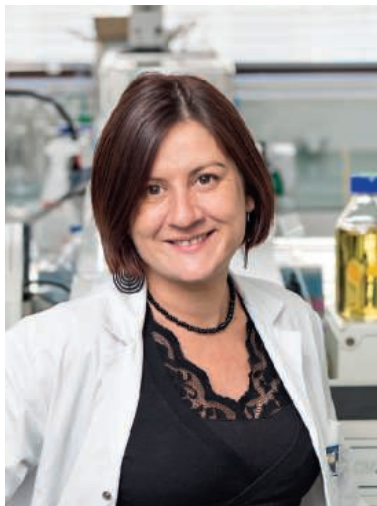
SANOFI - INSTITUT PASTEUR
2013 AWARDS
FOR
BIOMEDICAL RESEARCH

INSPIRED BY PASTEUR SUPPORTED BY SANOFI



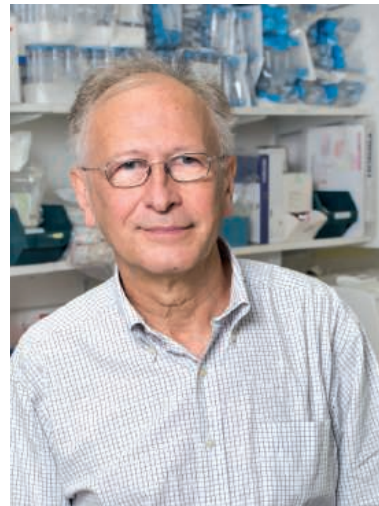
• **Dr. Carolina BARILLAS-MURY**
National Institutes of Health
(USA)

*Honored in the category of
"Tropical and neglected diseases".*



• **Dr. Christelle DESNUES**
Centre Hospitalier Universitaire
la Timone, CNRS (France)

*Honored in the category of
"Innovative approaches to disease
prevention, including vaccines".*



• **Pr. Alain FISCHER**
Hôpital Necker-Enfants
malades, Inserm,
Université Paris Descartes (France)

*Honored in the category of
"Immunology, immunomodulation,
immunogenetics and translational
technologies".*



• **Pr. Roy KISHONY**
Harvard Medical School
(USA)

*Honored in the category of
"New approaches to drug resistance".*

The Sanofi - Institut Pasteur 2013 Awards for Biomedical Research recognize four scientists whose research has achieved outstanding progress in the life sciences. The laureates, selected by a distinguished international jury, received their Awards Tuesday, November 5th, 2013 in Paris.

More information at:
www.sanofi-institutpasteur-awards.com

Luminex® Multiplex Immunoassays from R&D Systems

Analyze cancer serum samples:

Inflammation markers	Angiogenesis factors	Matrix remodeling factors
Angiopoietin-2	Angiogenin	EMMPRIN
IL-1 α	Angiopoietin-1	MMP-1
IL-1 β	Endostatin	MMP-2
Chitinase 3-like 1	FGF acidic	MMP-3
Cripto-1	FGF basic	MMP-7
DcR3	PDGF-AA	MMP-9
IL-12 p70	PDGF-BB	TIMP-1
IL-17A	PIGF	TIMP-2
IL-18 BP α	+ VEGF	TIMP-3
+ TNF- α	Luminex Assay	+ TIMP-4
Luminex Assay		Luminex Assay

Exclusively available from R&D Systems

One simple solution for profiling your complex samples

The world leader in the development of single analyte immunoassays now offers the widest selection of Luminex bead-based multiplex kits. Several features of our Luminex products distinguish us from the competition:

- We uniquely offer two different types of Luminex Kits: Luminex Screening & Luminex Performance Assays.
- We have the widest selection of analytes with over 20% of our menu being exclusively available from R&D Systems -160 screening analytes, 16 performance panels.
- We have developed the simplest online ordering tool for researchers to design their own user-defined kits.

Find your multiplex solutions

| RnDSystems.com/Luminex



Luminex is a registered trademark of Luminex Corporation.

There's only one

Science



Science Careers Advertising

For full advertising details, go to ScienceCareers.org and click For Employers, or call one of our representatives.

Tracy Holmes

Worldwide Associate Director
Science Careers
Phone: +44 (0) 1223 326525

THE AMERICAS

E-mail: advertise@sciencecareers.org
Fax: 202-289-6742

Tina Burks

East Coast/West Coast/South America
Phone: 202-326-6577

Marci Gallun

Midwest/Canada
Phone: 202-326-6582

Candice Nulsen

Corporate
Phone: 202-256-1528

Online Job Posting Questions

Phone: 202-312-6375

EUROPE / INDIA / AUSTRALIA / NEW ZEALAND / REST OF WORLD

E-mail: ads@science-int.co.uk
Fax: +44 (0) 1223 326532

Axel Gesatzki

Phone: +44 (0)1223 326529

Sarah Lelarge

Phone: +44 (0) 1223 326527

Kelly Grace

Phone: +44 (0) 1223 326528

JAPAN

Yuri Kobayashi

Phone: +81-(0)90-9110-1719
E-mail: ykobayas@aaas.org

CHINA / KOREA / SINGAPORE / TAIWAN / THAILAND

Ruolei Wu

Phone: +86-1367-1015-294
E-mail: rwu@aaas.org

All ads submitted for publication must comply with applicable U.S. and non-U.S. laws. Science reserves the right to refuse any advertisement at its sole discretion for any reason, including without limitation for offensive language or inappropriate content, and all advertising is subject to publisher approval. Science encourages our readers to alert us to any ads that they feel may be discriminatory or offensive.

Science Careers

From the journal Science



ScienceCareers.org



Massachusetts
Institute of
Technology

Come work with us!

Faculty Position

Department of Materials Science and Engineering

The Department of Materials Science and Engineering (DMSE) seeks candidates for two open tenure-track faculty positions to begin July 2014 or thereafter. Appointments would be at the assistant or untenured associate professor level. In special cases, a senior faculty appointment may be possible. Faculty duties include teaching at the graduate and undergraduate levels, research, and supervision of student research.

Candidates should hold a Ph.D. in Materials Science and Engineering or a related field by the start of employment. Candidates with deep knowledge of the core of Materials Science and Engineering are desired. DMSE seeks to broaden its research portfolio in two areas:

- Electronic and photonic materials. Some topics of interest include: multifunctional materials with cross-properties; photonic materials and photonic/electronic integration; hybrid electronic systems; materials theory for electronic and photonic materials.
- Materials chemistry and structure. Some topics of interest include: multiscale characterization science; low-dimensional materials; surface science; materials chemistry; materials theory.

However, DMSE has strengths and interests across the full spectrum of materials research, and excellent candidates with expertise in **any and all areas of the field** are welcomed.

MIT has a number of Institute-wide initiatives under way or in development, on topics that include Manufacturing, Energy, Environment, and Health. Individuals who can connect to these initiatives are of interest.

Interested candidates should submit application materials electronically at <http://dmsefacsrch.mit.edu>. Each application should include: a curriculum vitae; a statement of research interests; and a statement of teaching interests.

We request that each candidate arrange for 3 letters of reference to be uploaded at <http://dmsefacsrch.mit.edu/letters/>. Questions should be addressed to DMSE-Search-Master@dmsefacsrch.mit.edu. Responses received by December 31, 2013, will be given priority. No application received after March 1 will be considered in this year's search.

We especially encourage minorities and women to apply because of MIT's strong commitment to diversity in engineering education, research and practice.

MIT is an Equal Opportunity/Affirmative Action employer.

<http://web.mit.edu>



Institute for Systems Genetics

New York, NY

The newly formed **Institute for Systems Genetics at NYU Langone Medical Center** invites applications for both **tenured or tenure-track faculty positions** at the **assistant professor, associate professor, and full professor level**. The Institute, to be headed by Jef Boeke, Ph.D., will combine the latest technologies in Systems, Sequence and Synthetic Biology to tackle fundamental and translational questions in Genetics and Epigenetics. The Institute's Research interdisciplinary programs will center on four general overlapping areas: Human Genetics; 'Omics technologies and their application; Computational Sciences; and Biological Engineering - all practiced in a highly collaborative environment. The Institute will occupy new space in the Alexandria Center for Life Sciences West Tower on the East Side of Midtown/Lower Manhattan. The space is designed to foster highly collaborative and innovative research, and operate side-by-side with new initiatives in metabolism, microbiology, and immunology to complement the vibrant overall biomedical research environment at NYU Langone Medical Center, as well as the growing 'Omics community in New York City. State-of-the-art genomics, proteomics, and robotics facilities will be integral components of the Institute, and will leverage outstanding existing core facilities at NYU School of Medicine. Collaborations with the recently founded New York Genome Center and related entities will be encouraged. We seek senior and junior colleagues with hypothesis-driven research programs that exploit genetic, 'Omic, engineering and/or computationally intensive methods. Technology developers are also welcome. Candidates in the following areas are especially relevant:

- Human Genetics and Genomics
- Computational Biology
- Functional Genomics
- High throughput imaging or phenotyping
- Proteomics
- Engineering Biology

Candidates from nontraditional backgrounds such as Computer Science, Engineering, Physics, Chemistry and Mathematics are encouraged to apply. The successful candidate will hold a primary appointment in one of the more relevant NYU School of Medicine Departments, and will have opportunities to participate in one or more graduate programs.

Candidates should send a CV, research plan, and the name, telephone number and email address of four references (consolidated into a single pdf document) by **1/31/14**, to: ISGNYUjobs@gmail.com. Please have references send letters of recommendation as pdf documents. Senior faculty members are not obligated to request letters.

NYU Langone Medical Center is an Equal Opportunity Employer.



**SAN DIEGO STATE
UNIVERSITY**

Leadership Starts Here

FACULTY POSITIONS

ASSISTANT OR ASSOCIATE PROFESSORS IN VIROMICS

San Diego State University is a leader in the metagenomics, ecology, bioinformatics, and genetics of viruses, particularly phages. Four new colleagues will be hired over the next 2 years. Successful candidates will join the recently established interdisciplinary Viral Information Group (Edwards, Rohwer, Salamon, and Segall). The goal of these hires is to create a highly creative, collaborative working group interested in viruses, particularly but not exclusively viruses of microbes, from the molecular to the ecosystem level. Successful candidates will interact with the Viral Information Group, Center for Microbial Sciences, Molecular Biology Institute, Computational Science Research Center, and colleagues across campus.

Genome Engineer/Synthetic Biologist – The Department of Biology seeks a scientist who will rationally modify organisms, for example by building operons, pathways, and/or genomes, employing modern genetic engineering methods and developing new approaches that permit testing hypotheses about biological processes and gene function predictions. Electronic applications should be sent to genoeng@mail.sdsu.edu (see below). Full description is found at <http://www.bio.sdsu.edu/jobs/>.

Biomathematical modeler – The Department of Mathematics and Statistics seeks a theorist with strong foundations in computational methods and the hard sciences and have demonstrated ability to apply these methods to modeling biological processes at the community, population, biochemical, and/or biophysical level, and develop novel probabilistic approaches and dynamical modeling techniques to predict new functional features of existing and newly engineered organisms. Electronic applications should be sent to biomath@mail.sdsu.edu (see below). Full description is found at <http://www.math.sdsu.edu/jobops/>.

Biological Physicist – The Department of Physics seeks a biophysicist who uses optics and/or microfluidics to study reactions at the single cell or single molecule level. Desired approaches would include using single cell genomics to identify virus-host systems and using microfluidics and high throughput methods to dissect unknown protein function. Electronic applications should be sent to biophysics@mail.sdsu.edu (see below). Full description is found at <http://www.physics.sdsu.edu/job-opportunities/>.

Structural Biologist – The Department of Biology seeks a colleague who will employ physical and/or computational modeling techniques in the structural study of biological macromolecules and macromolecular interactions of viruses and host cells and/or viral-dependent modifications of host cells. Methods could include X-ray crystallography, cryo-electron microscopy, computational structural modeling, NMR microscopy for functional screens. Electronic applications should be sent to structbio@mail.sdsu.edu (see below). Full description is found at <http://www.bio.sdsu.edu/jobs/>.

ASSISTANT PROFESSOR IN EVOLUTIONARY GENOMICS/GENETICS

The Department of Biology seeks a creative, productive evolutionary biologist with research strengths in eukaryotic genomics or genetics. We are especially interested in candidates who use innovative experimental, computational and/or comparative approaches. Research area is open to studies of all eukaryotic organisms above or below the species level. Preference will be given to those candidates with a central focus in evolutionary biology who also clearly demonstrate cross-disciplinary research programs.

Candidates should have post-doctoral experience and a strong record of research accomplishments and funding. The successful candidate will participate in the MS and PhD programs in Evolutionary Biology and may also participate in the department's MS and PhD programs in Cell and Molecular Biology, and Ecology. Electronic applications should be sent to evgenomics@mail.sdsu.edu (see below). Full description is found at <http://www.bio.sdsu.edu/jobs/>.

ASSISTANT OR ASSOCIATE PROFESSOR IN ECOSYSTEM AND LAND SURFACE MODELING

San Diego State University is a leader in climate change impacts on ecosystems and feedbacks to ecosystems from climate change and has strength in sustainability, climate modeling, and interactions of civilizations and climate. Four new colleagues will be hired over the next 2 years. In addition to this position, we are seeking a climate statistician (Mathematics; full description at <http://www.math.sdsu.edu/jobops/>). Next year we will seek a regional climate modeler and an archaeologist working with the interaction of climate and civilizations. The successful candidates will join the recently established interdisciplinary Center on Climate and Sustainability Studies (C²S²) (Oechel, Shen, Lipson, Lai, Biggs, Braje, Lauer). Our goal is to create a highly productive, interactive center working on climate causes and feedbacks at all scales. The successful candidate will interact with the Center for Climate and Sustainability Studies, the Ecology Program Area, Math and Statistics, and colleagues across campus.

Ecosystem modeler - The Department of Biology seeks an Assistant or Associate level with expertise in modeling **ecosystem and/or biosphere-atmosphere interactions** who will collaborate with colleagues in carbon cycling, climate statistics, water resources, and human-environment interactions. Demonstrated experience in running dynamic vegetation models (DVM) and land surface models (such as NCAR CLM) to quantify biospheric feedbacks to the climate system are desirable. Electronic applications should be sent to ecosystem@mail.sdsu.edu (see below). Full description is found at <http://www.bio.sdsu.edu/jobs/>.

For each position, candidates must have a Ph.D. in an appropriate field; postdoctoral experience is preferred. Demonstrated research potential through a record of publications and funded grants is expected. Successful candidates will participate in one or more Master's and Joint Doctoral programs, and will contribute to SDSU's undergraduate and graduate teaching mission. Send a single pdf with a cover letter, curriculum vitae, statement of research, statement of teaching, and representative publications to the appropriate email. Review of applications will begin December 16, 2013 for the Viromics and Evolutionary Genomics positions, and January 18, 2014 for Ecosystem Modeling. Review will continue until the positions are filled.

SDSU is an Equal Opportunity/Title IX Employer.



COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK

The 2014 Louisa Gross Horwitz Prize for Biology or Biochemistry

The Louisa Gross Horwitz Prize was established under the will of the late S. Gross Horwitz through a bequest to Columbia University and is named to honor the donor's mother. Louisa Gross Horwitz was the daughter of Dr. Samuel David Gross (1805-1889), a prominent surgeon of Philadelphia and author of the outstanding *Systems of Surgery* who served as President of the American Medical Association.

Each year since its inception in 1967, the Louisa Gross Horwitz Prize has been awarded by Columbia University for outstanding basic research in the fields of biology or biochemistry. The purpose of this award is to honor a scientific investigator or group of investigators whose contributions to knowledge in either of these fields are deemed worthy of special recognition.

The Prize consists of an honorarium and a citation which are awarded at a special presentation event. Unless otherwise recommended by the Prize Committee, the Prize is awarded annually. The 2013 awardee(s) will be announced in December 2013.

QUALIFICATIONS FOR THE AWARD

The Prize Committee recognizes no geographical limitations. The Prize may be awarded to an individual or a group. When the Prize is awarded to a group, the honorarium will be divided among the recipients, but each member will receive a citation. Preference will be given to work done in the recent past.

Nominations must be submitted electronically at: <http://www.cumc.columbia.edu/research/horwitz-prize>

All communications and materials must be written in the English language.

Re-nomination(s) are by invitation only.

Self-nominations are not permitted.

Nominations should include:

- 1) A summary, no more than 500 words long, of the research on which this nomination is based.
- 2) A summary, no more than 500 words long, of the significance of this research in the fields of biology or biochemistry.
- 3) A brief biographical sketch of the nominee, including positions held and awards received by the nominee.
- 4) A listing of up to ten of the nominee's most significant publications relating to the research noted under item 1.
- 5) A copy of the nominee's curriculum vitae.

Deadline date: January 31, 2014

MICHIGAN STATE UNIVERSITY

Rudolph Hugh Endowed Chair in Microbial Pathogenesis Department of Microbiology and Molecular Genetics

The Department of Microbiology and Molecular Genetics at Michigan State University (<http://www.mmg.msu.edu>) seeks candidates for the Rudolph Hugh Endowed Chair position at the Associate or Full Professor level in microbial pathogenesis. Applicants are sought with demonstrated expertise in the evolution of pathogenicity, bacterial pathogenesis, microbial ecology of infectious diseases, genetics of virulence, or host-microbe interactions; however, outstanding candidates in other related areas are encouraged to apply. A strong record of research accomplishment and an independent, externally funded research program with international visibility are required. The candidate will be expected to build and lead collaborative groups and to mentor junior faculty. Many opportunities exist to engage in collaborations through the BEACON Center for the Study of Evolution in Action, the Enteric Disease Research Investigational Network, the International Center of Excellence for Malaria Research, and Center for Microbial Ecology. Teaching within our graduate, professional, and/or undergraduate programs is expected. The offer will include a highly competitive startup package, laboratory facilities, and substantial annual discretionary funding from the Hugh Endowment.

Review of applications will begin immediately. The position will remain open until filled. Applicants must submit the following application items through the Human Resources (MAP) website at <https://jobs.msu.edu> (position #8642):

- (a) Letter of interest which should provide a statement of current and future research and plans
- (b) Curriculum vitae that includes a history of funding

In addition, applicants are asked to send the letter of interest and the names of three potential references (not to be contacted until approval is received from the applicant) via email to the Search Committee Executive Assistant at mmgchair@msu.edu. Questions regarding the position can be addressed to the chair of the search committee, **Dr. Martha Mulks**, at mulks@msu.edu.

MSU is committed to achieving excellence through cultural diversity. The University actively encourages applications of women, persons of color, veterans and persons with disabilities and endeavors to facilitate employment assistance to spouses or partners of candidates for faculty and academic staff positions. Michigan State University is an Affirmative-Action, Equal-Opportunity Employer.

SYRACUSE UNIVERSITY

Assistant Professor in Biochemistry (070778)

The Departments of Biology and Chemistry at Syracuse University are soliciting applications for the joint position of Assistant Professor of Biochemistry. The successful applicant will have a PhD in Chemistry, Biology or related areas and relevant postdoctoral experience. The candidate is expected to teach Biochemistry at all levels and develop a vigorous research program with strong extramural funding.

The candidate will find an enthusiastic research environment with excellent facilities and many opportunities for collaboration in Biology, Chemistry, and Biomaterials at Syracuse University, and the adjacent SUNY campuses of the Upstate Medical University and Environmental Science and Forestry. Competitive salary, start-up funds and laboratory space will be provided.

The College of Arts & Sciences is interested in candidates who have demonstrated commitment to excellence by providing leadership in teaching, research or service towards building an equitable and diverse scholarly environment. For details and to apply, go to www.sujobopps.com #070778.

Review of applications will begin December 2, 2013. For questions, please e-mail Philip Borer (pnborer@syr.edu; chair of the search committee).

Syracuse University is an
Affirmative Action/Equal Opportunity
Employer.



Senior Professorial Researcher in Regenerative Medicine, Australian Regenerative Medicine Institute

Monash is seeking expressions of interest from dynamic, experienced, stem cell-focused, regenerative medicine research scientists with outstanding research track records and a desire to work in a multidisciplinary environment.

The successful candidate will bring an internationally recognised track record of outstanding regenerative medicine and/or stem cell research with the ability to attract independent funding and will be expected to initiate and maintain strong research programs synergizing with the Monash research environment to take an active part in collaborative research. Clinician scientists are encouraged to apply.

To attract the right individual, this full professorial position will be offered for a five year period with a further five year extension upon successful review and will be made at a level appropriate to the candidate's qualifications and experience.

A cover letter, curriculum vitae, full publication list, a concise description of research interests and statement of previous research achievements and teaching merits, and a list of at least three reference persons should be provided to **Denise.Wangman@Monash.edu** by **31 December 2013**.

Established through a joint venture between Monash University and the Victorian Government, the Australian Regenerative Medicine Institute (ARMI) builds on the University's existing strengths in biomedical research, and supports the critical infrastructure required to deliver the next generation of discoveries in regenerative medicine.

ARMI is located at one of the world's largest regenerative medicine and stem cell research centres, at the Clayton (Victoria, Australia) campus of Monash University. Its scientists are focused on unravelling the basic mechanisms of the regenerative process, enabling doctors to prevent, halt and reverse damage to vital organs due to disease, injury or genetic conditions.

To learn more about ARMI and the work we do please visit **www.armi.org**.

Monash University is an energetic and dynamic university committed to quality education, outstanding research and international engagement. A member of Australia's Group of Eight research-intensive universities, it seeks to improve the human condition and is committed to a sustainable future. Monash has six campuses in Victoria, a campus in Malaysia, a campus in South Africa, a centre in Prato, Italy, and numerous international partnerships and cooperative ventures. Monash has over 62,500 equivalent full-time students spread across its Australian and off-shore campuses, and over 7,400 full time equivalent staff. Almost 3,500 of these staff members are academic staff.

Further information regarding this position is available from Professor Nadia Rosenthal, Director or Peter Currie, Deputy Director, Australian Regenerative Medicine Institute at **peter.currie@monash.edu**

Interested applicants should send their expression of interest by email to **Denise.Wangman@Monash.edu**

Deadline for applications is **31 December, 2013**.

SANTA FE INSTITUTE

BECOME A COMPLEXITY SCHOLAR

Because Our World Isn't Simple.

SCHOOLS

Complex Systems Summer School

June 2014, Santa Fe, New Mexico,
Graduate students & postdoctoral fellows

Graduate Workshop in Computational Social Science & Modeling

June 2014, Santa Fe, New Mexico, Graduate students

Complexity and Modeling Program (C.A.M.P)

July 2014, Massachusetts, High school students

FELLOWSHIPS & INTERNSHIPS

SFI Omidyar Fellowship

Ongoing, Postdoctoral fellows

Research Experiences for Undergraduates

June-August 2014, Santa Fe, New Mexico, Undergraduate students

Project GUTS: Growing Up Thinking Scientifically

After-school programs in selected communities, various dates
Middle school students

GUTS y Girls

After-school programs in selected communities, various dates
Female middle school students

PROFESSIONAL DEVELOPMENT

SFI Short Course on Complexity

September 2014 (three days), Professionals, no math or
science background required

New Mexico Computer Science for All

January – December 2014, High School teacher professional
development, program in computer science

ONLINE COURSES

Introduction to Dynamics

& Chaos Massive
Open Online Course
by Dave Feldman
January 2014

Agent-Based Modeling

with NetLogo Massive
Open Online Course
by Melanie Mitchell
April 2014

Introduction to

Complexity Massive
Open Online Course
by Melanie Mitchell
Fall 2014

Nonlinear Dynamics

Massive
Open Online Course
by Elizabeth Bradley
September 2014

Complexity Explorer

Online educational
resource for teachers
& students of
complexity science



**For eligibility,
application deadlines,
fees, tuition assistance
information, and
other details, visit
[www.santafe.edu/
education](http://www.santafe.edu/education)**



Director of the WWAMI Medical Education Program

The University of Idaho (UI) is seeking a Director for the WWAMI Medical Education Program. The WWAMI (Washington, Wyoming, Alaska, Montana, Idaho) Medical Education Program is the regional medical education program of the University of Washington School of Medicine (UWSOM) in Idaho.

The director is primarily responsible for assuring the excellence of the first year medical school program at the UI, actively participating in teaching the first year medical school courses, promoting research and other scholarly activities with the opportunity to continue an active research program and participate in graduate education, and budgeting funds allocated to the programs from state, private and federal sources.

Minimum requirements for this position include a terminal degree (Ph.D., M.D., or equivalent) in a discipline relevant to biomedical sciences or medical education, experience in teaching medical students, and a demonstrated aptitude for management of budgets, personnel and other resources.

To apply, applicants must complete the online application available at the University of Idaho's Human Resources site: (<http://apptrkr.com/402942>). Additional information for candidates is available at the Provost and Executive Vice President site: (<http://www.uidaho.edu/provost/deansearches/>).

Questions regarding the position, search process, or candidate nominations may be directed to the search advisory committee chair, Dr. Joe Cloud, Interim Director of WWAMI, jcloud@uidaho.edu or (208) 885-6696, or the search coordinator and Assistant to Director, Marlane Martonick, marlanem@uidaho.edu, or (208)-885-6696.

To enrich education through diversity, the University of Idaho is an Equal opportunity/Affirmative Action Employer.



TEXAS BIOMEDICAL
RESEARCH INSTITUTE



SNPRC
Southwest National
Primate Research
Center

STEM CELL REGENERATIVE MEDICINE FACULTY POSITIONS Southwest National Primate Research Center (SNPRC)

The SNPRC at the Texas Biomedical Research Institute invites applications and nominations for two faculty positions in stem cell regenerative medicine. Applicants and nominees are expected to have an interest in, and preferably experience with, nonhuman primate models. Major strengths of the SNPRC are in genetics and genomics, metabolic disorders, and infectious diseases. Several primate models of human diseases developed at SNPRC are ideally suited for translational research on stem cell regenerative medicine. The SNPRC has an outstanding diversity of primate resources, including large colonies of baboons, rhesus monkeys, common marmosets, and chimpanzees.

The SNPRC has close associations with the University of Texas Health Science Center at San Antonio and the University of Texas at San Antonio, including opportunities for roles in graduate education. A critical mass of stem cell regenerative medicine researchers exists at those institutions, together with the developing stem cell regenerative medicine program at the SNPRC. The SNPRC has a strong tradition in postdoctoral training.

Rank and salary will be nationally competitive and commensurate with experience. The laboratories and offices of the successful candidates will be located in the new SNPRC laboratory and administration facility, which is now under construction and expected to be ready for occupancy in March 2014.

Applications and nominations should be sent to the **Chair, SNPRC Regenerative Medicine Search Committee, c/o Human Resources Office, P. O. Box 760549, San Antonio, TX 78245-0549**, and should include a letter outlining qualifications and research interests. Applications, but not nominations, must also include a CV, and the names and contact information for at least three references. Additional information about the SNPRC can be found at www.snprc.org. Additional information about Texas Biomed can be found at www.txbiomed.org. *EOE*



PRINCETON
UNIVERSITY

ASSISTANT PROFESSORSHIP QUANTITATIVE EVOLUTIONARY GENETICS

Princeton University's Department of Ecology & Evolutionary Biology and the Lewis-Sigler Institute for Integrative Genomics seek to jointly hire a tenure-track Assistant Professor focusing on Evolutionary and Quantitative Biology. Sample areas might include, but are not limited to: molecular/genome evolution, population genomics, evolution of development, behavioral genetics, experimental evolution, microbial evolution of prokaryotes or eukaryotes, epigenetics, metagenomics, and/or quantitative genetics, using traditional and/or emerging model systems (though the specific model system is less important than the nature of the questions being addressed). We seek applicants who pursue research that aims for significant conceptual integration across traditional disciplinary boundaries. We likewise seek colleagues who will enthusiastically contribute to a climate that embraces both excellence and diversity, and who share our commitment to a mentoring process that advances EEB, LSI and the university, and that attracts and retains students of all ethnicities, nationalities, and genders.

Applicants should write a vision statement, no longer than 2 pages, that outlines one or more major unsolved problems in their field and how they plan to address them. *In this respect, the vision statement should go beyond just a summary of the applicant's prior and current research.* Applications, including a cover letter with links to three major publications or pre-prints, the vision statement, curriculum vitae, and contact information of three references for online reference request, must be submitted online via <http://jobs.princeton.edu>, to Req #1300612. Screening of applications will begin immediately and continue until the position is filled.

Princeton University is an Equal Opportunity Employer and complies with applicable EEO and Affirmative Action regulations.

THE
GRADUATE
CENTER

CITY UNIVERSITY
OF NEW YORK

Follow your dreams. Achieve your goals.

**Study with world-class
faculty mentors and scholars
in state of the art research
facilities.**

The Ph.D. programs in **Biochemistry, Biology, Chemistry, and Physics**, award CUNY Science Scholarships that include a \$25K annual stipend, tuition, and affordable health coverage for up to five years. Come study with us.

For more information visit:
www.gc.cuny.edu or
scan the QR code.



LUNDBECK FOUNDATION FELLOWSHIPS

The Lundbeck Foundation hereby invites applications for five fellowships within biomedicine which will be granted to particularly promising young researchers.

The fellowships are awarded for five years and each fellowship amounts to DKK 10 million (approx. Euro 1,3 million).

The subject area should be frontline basic- or applied research within the scope of the Foundation's grant strategy, which can be seen at www.lundbeckfonden.com

The fellowships may well attract Danish or foreign researchers from abroad, who wish to move to Denmark and continue their research here. The call is also open for applicants at Danish universities and university hospitals.

The fellowships are intended for five researchers who are qualified to establish or develop their own research groups within biomedicine and who have received their Ph.D. degree within the last 5-7 years.

The application should include an account of the research plan, collaborators, budget and how the research group is envisioned to be placed within a Danish research institution. In addition, it should include a letter of intent from a resident researcher at the host institution, who makes him- or herself available as a mentor to facilitate the applicant's establishment of the research group as an integral part of the host institution. Further guidance is provided in the application form.

The application, written in English, should be sent via the Foundation's Electronic Application System for fellowships at www.lundbeckfonden.com no later than December 16, 2013.

For further information please contact Ulla Jakobsen, Science Manager at the Lundbeck Foundation, phone: +45 39 12 80 11 or at application@lundbeckfonden.com



The Lundbeck Foundation has controlling shareholdings in its subsidiaries H. Lundbeck, ALK and Falck. In addition, the Foundation manages financial investments of approx. € 1.5 billion. The Foundation supports biomedical research. In 2012, the Foundation had a profit after tax of approx. € 277 million and made research grants of approx. € 65 million.

Lundbeckfonden
Vestagervej 17, DK-2900 Hellerup
Tel. +45 39 12 80 00
www.lundbeckfonden.com

THE LUNDBECK FOUNDATION



Director and Chief Executive ROTHAMSTED RESEARCH

Lead an internationally renowned Institute with a key role to play in combating the global challenges of food security and environmental change.

At the forefront of both fundamental and translational research, Rothamsted Research is a centre of international scientific excellence in support of sustainable land management and agriculture and their environmental impacts with particular relevance to crops, including grassland and products from crops; soil and soil management practices and the diverse interactions that occur between plants, farming practices, other organisms and the physical environment.

You will provide inspirational leadership and vision to a multi-disciplinary team of over 350 staff, plus PhD students and visiting scientists, ensuring that the Institute both maintains and develops its position and reputation as a world-class organisation, playing a key role in the delivery of discovery and strategic programme level research that can be used by a range of different stakeholders, including Government and industry, nationally and internationally.

The post will be based at the Rothamsted Research main campus in Harpenden, Hertfordshire.

An attractive salary plus performance related bonus is available commensurate with the level and strategic importance of the post.

Further information, including instructions on how to apply, may be obtained from Rothamsted Research's recruitment consultants, Carbon-NfP, who would be happy to have an informal and confidential telephone discussion. Please contact Sharon Taylor by email sharon.taylor@carbon-nfp.com in the first instance.

The closing date for applications is Friday, 13 December 2013.

Rothamsted Research is a registered charity (no. 802038) Limited by guarantee and is an Equal Opportunities Employer

www.carbon-nfp.com



ASU[®] SCHOOL OF Life Sciences

ARIZONA STATE UNIVERSITY

Assistant Professor (JOB# 10594)

The School of Life Sciences at Arizona State University invites applications for a tenure-track faculty position at the level of Assistant Professor in the area of microbial genomics including metagenomics. Anticipated start date is August 16, 2014. We encourage applications from outstanding candidates who employ an integrated and innovative basic or applied research approach leading to important contributions related to Microbial Genomics. Candidates who focus on a relevant area within Microbial Genomics, including metagenomics, physiology, genetic engineering, biotechnology, bioinformatics, bioprospecting, evolution, microbial interactions, or microbial ecology are preferred. The successful candidate will be expected to develop a strong, extramurally funded independent research program; teach at the undergraduate and graduate levels; successfully mentor undergraduate and/or graduate students, and postdoctoral associates; and actively engage in outreach and service within and outside the University. A competitive start-up package and teaching load compatible with high research productivity will be provided.

Arizona State University has a vibrant, interdisciplinary research and education community that the successful candidate will become part of. Examples of relevant centers and initiatives include the Bioenergy and Photosynthesis Center (<http://bioenergy.asu.edu>), Biodesign (<http://www.biodesign.asu.edu/>), and Astrobiology (<http://astrobiology.asu.edu>). Candidates must have a Ph.D. (or equivalent) in microbiology, genomics or another appropriate field. Two years of postdoctoral training, teaching experience, and a record of accomplishment that illustrates strong preparedness for establishing an excellent, independent research program in Microbial Genomics is preferred.

To apply, send a cover letter, curriculum vitae, three representative publications, contact information for at least three references, and separate statements of future research plans and teaching philosophy interests in a single pdf file to solsfacultysearch2@asu.edu. The initial closing date for receipt of applications is **December 1, 2013**; applications will be reviewed weekly thereafter until the search is closed. A background check is required for employment.

Arizona State University is an Equal Opportunity/Affirmative Action Employer committed to excellence through diversity. Women and minorities are encouraged to apply. For additional information on this position and the School of Life Sciences, please visit <http://sols.asu.edu/jobs>.

Tenure-track Assistant Professor, Department of Biological Sciences

Needs exist in: human anatomy, physiology, integrative biology, and organismal introductory biology. Ph.D. in a biological sciences discipline, completed by August 31, 2014; broad training and/or experience in biological sciences; experience in college teaching and in working with diverse groups required. Preference given to applicants who are willing to help the department develop innovative teaching strategies. The Department of Biological Sciences at California State University, Sacramento expects its faculty members to achieve excellence in classroom teaching; they are also expected to engage in scholarship, student advising, university service, and community service. Mail CV, all transcripts, email addresses and telephone numbers of three references, statements of teaching and scholarly interests, and three letters of recommendation to: **Jennifer Lundmark, Chair, Biological Sciences, California State University, Sacramento, CA 95819-6077**. Website: <http://www.csus.edu/bios/>. To ensure full consideration, all application materials should be received by **December 16, 2014**; position open until filled. For more detailed information, please see vacancy announcement at <http://www.csus.edu/hr/facultyvacancies/vacancies.html>.

Equal Opportunity Employer. Clery Act statistics available. Mandated reporter requirements. Criminal background checks may be required.

ASU[®] SCHOOL OF Life Sciences

ARIZONA STATE UNIVERSITY

Assistant Professor (JOB# 10593)

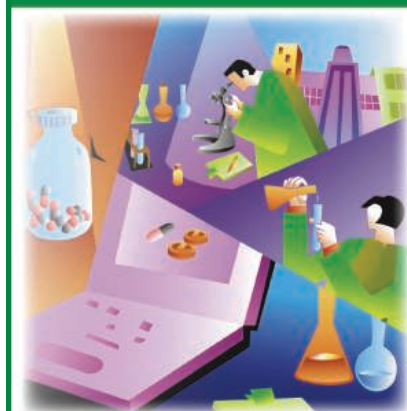
The School of Life Sciences and The Biodesign Institute at Arizona State University invite applications for a tenure-track faculty position at the level of Assistant Professor whose research focuses on comparison of biological systems at the genome scale. Anticipated start date is August 16, 2014. Preferred research methods may include but are not limited to theoretical, computational, populational, and empirical approaches to comparative and functional genomics. The successful candidate will be expected to develop an innovative, extramurally-funded, research program, teach at the undergraduate and graduate levels, and have a commitment to outreach and service. The successful candidate will be expected to mentor undergraduate and graduate students as well as postdoctoral fellows. A competitive start-up package and teaching load compatible with high research productivity will be provided.

Arizona State University has made a commitment to accelerating the translation of basic discoveries into practical benefits for society through the construction of state-of-the-art research facilities and the recruitment of world-class faculty members. The successful candidate will participate in university-wide health and/or sustainability initiatives supported by core facilities for functional genomics and next generation sequencing, functional proteomics, high throughput cellular screening, bioinformatics, high performance computing, and imaging. More information on genomic research opportunities at the Biodesign Institute and the School of Life Sciences at ASU can be found at <http://genomics.asu.edu>. Candidates must have a Ph.D. (or equivalent) in an appropriate field. Demonstrated teaching and research excellence is preferred.

To apply, send cover letter, your curriculum vitae, three representative publications, separate statements of future research plans and teaching philosophy and interests, and contact information for three references to be sent to **Kenro Kusumi, Chair, Comparative Genomics Faculty Search Committee, School of Life Sciences, PO Box 874501, Tempe, AZ 85287-4501**. Electronic applications sent as PDF files to solsfacultysearch@asu.edu are preferred. The initial closing date for receipt of applications is **November 25, 2013**; applications will be reviewed every two weeks thereafter until the search is closed. A background check is required for employment.

Arizona State University is an Equal Opportunity/Affirmative Action Employer committed to excellence through diversity. Women and minorities are encouraged to apply. For additional information on this position and the School of Life Sciences, please visit <http://sols.asu.edu/jobs>.

CAREER TRENDS Running Your Lab



Download your free copy today at ScienceCareers.org/booklets

Science Careers

From the journal *Science*



Brought to you by the
AAAS/Science Business Office



**Division of Research and Economic Development
Position of Director
Office of Sponsored Research and Programs**

Division of Research and Economic Development invites applications for the position of Director, Office of Sponsored Research and Programs (OSRP), which reports to the Vice Chancellor for Research and Economic Development. The OSRP proactively facilitates the pre-award process and oversees certain aspects of post-award grants management. Pre-award activities include providing technical assistance to faculty and staff to ensure continuity in the submission of proposals for grants and contracts to potential funding agencies. Post-award activities include providing regulatory oversight and complete, accurate, and timely financial information pertaining to contract and grant transactions to the University, funding agencies and other external stakeholders.

Master's degree required with a minimum of ten (10) years of experience working with sponsored programs/grants/contracts at a university and/or state and federal levels.

The Division anticipates filling the Position of Director, Office of Sponsored Research and Programs by February 1, 2014; however, review of applications will commence immediately and continue until the position is filled. Applications and/or Nominations may be submitted to: **Ms. Carol J. Hicks, Executive Assistant, Division of Research & Economic Development, Hubbard-Totton Building, Suite 309, North Carolina Central University, 1801 Fayetteville Street, Durham, NC 27707**; or via email cburne17@ncu.edu.

FUNDING OPPORTUNITIES

Department of Defense

**Defense Medical Research and Development Program
Clinical and Rehabilitative Medicine Research Program**

The Clinical and Rehabilitative Medicine Research Program (CRM RP) focuses on definitive and rehabilitative care innovations required to reset our wounded warriors, both in terms of duty performance and quality of life. The CRM RP encourages researchers to submit applications for the following upcoming funding opportunities.

Deadline for pre-applications: November 25, 2013

Neurosensory Research Award: Supports both applied (preclinical) research and clinical trials within specific focus areas of pain management, hearing loss/dysfunction, balance disorders and/or tinnitus. Applications focused on traumatic brain injury are highly encouraged.

Neuromusculoskeletal Injuries Research Award: Supports preclinical research and clinical trials on the functional utility of assistive devices related to the human-device interface, secondary health effects following injury, and optimizing rehabilitation and device prescription for patients with severe extremity trauma.

Regenerative Medicine Clinical Trial Award: Supports clinical trials focused on extremity regeneration, craniomaxillofacial regeneration, vascularized composite allografts, and/or genitourinary/lower abdomen reconstruction.

Vision Research Program Hypothesis Development Award: Supports conceptually innovative, high-risk/high-reward research that could ultimately lead to critical discoveries or major advancements that will drive the field of vision research forward.

Vision Research Program Translational Research Award: Supports translational research that will accelerate the movement of promising ideas in vision research into clinical applications.

All applications must conform to the final Program Announcements and General Application Instructions that will be available for electronic downloading from the Grants.gov website (all viewable under CFDA number 12.420). Execution management support will be provided by the Congressionally Directed Medical Research Programs (CDMRP) and the Telemedicine & Advanced Technology Research Center (TATRC).

<https://crmrp.amedd.army.mil/>
<http://cdmrp.army.mil>

<http://www.tatrc.org/>
<http://grants.gov>

connect collaborate cure

World Stem Cell Summit 2013

**December 4 – 6
San Diego, California**

Your Window to the World of Regenerative Medicine

- Make new contacts, build your network
- Forge relationships
- Discover what's new
- Showcase your work

**Poster abstracts due
November 15**

Find out more at www.worldstemcellsummit.com

Genetics
Policy
Institute

GPI.

CIRM

CALIFORNIA
INSTITUTE FOR REGENERATIVE MEDICINE
The State Stem Cell Agency

MAYO
CLINIC

THE
SCRIPPS
RESEARCH
INSTITUTE®



Sanford Burnham
Medical Research Institute



WSCS13

PURDUE UNIVERSITY

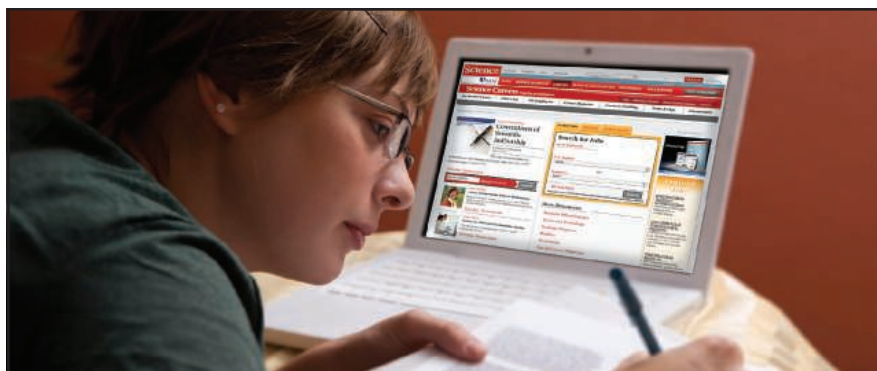
Purdue University College of Engineering and College of Science Faculty Openings in Integrated Imaging

Purdue University's College of Engineering and College of Science have identified imaging as a major thrust for cross-disciplinary research and education and are accepting applications for multiple tenured and tenure track faculty (Assistant and Associate Professor) in this area. Imaging research has become the key to progress in fields such as medicine, biology, chemistry, physics, earth and atmospheric science; and the resulting technologies are addressing major human needs in healthcare, medicine, security, manufacturing, and communication. Purdue University has embarked on a new "integrated-imaging" initiative to exploit the synergy between science and engineering, algorithms and devices, sensors and applications. This initiative will build on strengths and leverage the imaging capabilities of both Purdue Colleges and Purdue's Discovery and Research Parks.

Candidates must hold a Ph.D. degree in Engineering, Science or a related field. They should have a distinguished academic record, exceptional potential for world-class research, and a commitment to teach in both undergraduate and graduate programs. Specific research fields of interest in the integrated-imaging cluster include, but are not limited to electron and light microscopy, whole body imaging, image processing, inverse methods, multimodal imaging, hyperspectral imaging, and sensor systems. The successful candidate will teach undergraduate and graduate courses in topics related to integrated imaging, conduct research in their field of expertise, publish and present research findings, participate in professional activities, and advise graduate student research. The primary faculty appointment will be in a school of the College of Engineering or a department of the College of Science, and will depend on the candidate's qualifications.

Submit your application online at: <https://engineering.purdue.edu/Engr/InfoFor/Employment>. The application should include a cover letter, a complete and detailed vitae, and statements of research and teaching interests. Also, please include names, addresses, telephone numbers, and e-mail addresses for three or more references. For questions regarding the application process, please contact **Marion Ragland** (ragland@purdue.edu). Screening of applications will begin **December 1, 2013** and will continue until the positions are filled. A background check will be required for employment in this position.

Purdue University is an Equal Opportunity/Equal Access/Affirmative Action Employer fully committed to achieving a diverse work force.



AAAS is here – helping scientists achieve career success.

Every month, over 400,000 students and scientists visit ScienceCareers.org in search of the information, advice, and opportunities they need to take the next step in their careers.

A complete career resource, free to the public, *Science Careers* offers a suite of tools and services developed specifically for scientists. With hundreds of career development articles, webinars and downloadable booklets filled with practical advice, a community forum providing answers to career questions, and thousands of job listings in academia, government, and industry, *Science Careers* has helped countless individuals prepare themselves for successful careers.

As a AAAS member, your dues help AAAS make this service freely available to the scientific community. If you're not a member, join us. Together we can make a difference.

To learn more, visit
aaas.org/plusyou/sciencecareers



POSITIONS OPEN

UIC Center for Cardiovascular Research COLLEGE OF MEDICINE

TENURE-TRACK FACULTY POSITION

Center for Cardiovascular Research University of Illinois at Chicago College of Medicine

The University of Illinois at Chicago (UIC) Center for Cardiovascular Research (CCVR) in the College of Medicine seeks outstanding faculty candidates with expertise in key areas of cardiovascular science, with an emphasis on, but not limited to cardiac mitochondrial biology, metabolism, epigenetics, and noncoding RNA. Consideration will be given to applicants at all ranks (**ASSISTANT, ASSOCIATE, FULL PROFESSORS**).

Successful candidates will demonstrate the ability to garner extramural grant support and are expected to lead comprehensive and innovative research programs that focus on heart failure, diabetes, obesity, metabolic syndrome, and hypertension. A commitment to excellence in teaching is also required. Minimum degree requirements are a Ph.D. or M.D. degree with three or more years of postdoctoral training. Attractive startup packages are available, commensurate with experience.

Applicants will submit a letter of interest, stating a research plan, curriculum vitae, and names of at least three references. For more information on the CCVR, visit website: <http://www.ccvr.uic.edu>.

Apply online only at website: <https://jobs.uic.edu/job-board/job-details?jobID=32073&job=assistant-associate-professor-ccvr>.

UIC is an Affirmative Action/Equal Opportunity Employer.

FACULTY POSITIONS in Global Change Sciences

The University of California, Merced, Environmental Systems Graduate Group invites applicants for three, tenure-track academic positions as part of a Global Change Sciences Cluster Hire: Evolutionary Biology (School of Natural Sciences, **ASSISTANT PROFESSOR**), Ecological Theory/Modeling (School of Natural Sciences, **ASSISTANT PROFESSOR**), and Environmental/Ecological Engineering (School of Engineering, **OPEN RANK**). The ES Graduate Group brings together a diverse group of faculty, graduate students, and research scholars with a shared interest in interdisciplinary research of natural and human-impacted environmental systems. For more information and to apply, visit website: <http://jobs.ucmerced.edu/n/academic/listings.jsf?sessionId=4DD66CBAC92250789897AAE4EA75CABB&seriesId=1>.

The application deadline is January 10, 2014. *Affirmative Action/Equal Opportunity Employer.*

POSTDOCTORAL ASSOCIATES

The Institute of Marine and Coastal Sciences at Rutgers University is seeking Postdoctoral Associates in the areas of biological, chemical, geological, and physical oceanography. Prospective candidates are expected to identify and pursue creative research avenues within existing research programs and faculty expertise. These fellowships are one-year renewable appointments. Applications are presently being accepted and evaluated on an ongoing basis. However, applications received by December 15, 2013 will receive the fullest attention. To apply, please e-mail curriculum vitae, statement of research interest, and names of three references to **Dr. Richard A. Lutz** (Director, Institute of Marine and Coastal Sciences) at e-mail: postdocsearch@marine.rutgers.edu (please include "Postdoc" in the subject line).

Rutgers is an Equal Opportunity/Affirmative Action Employer.

Get your questions answered.
Careers Forum
www.ScienceCareers.org